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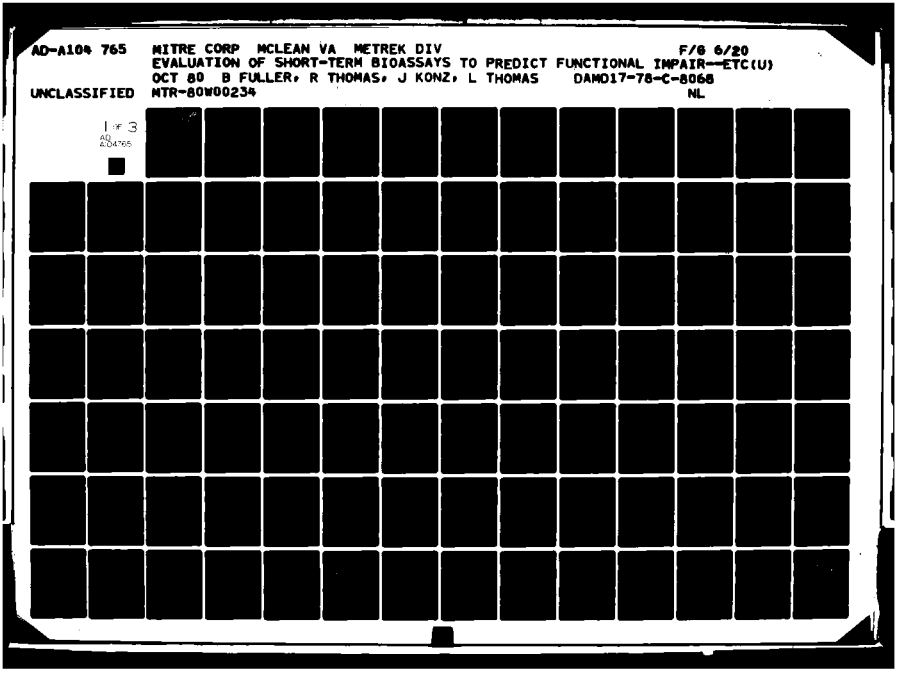

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EVALUATION OF SHORT-TERM BIOASSAYS TO PREDICT FUNCTIONAL IMPAIRMENT

SELECTED SHORT-TERM RENAL TOXICITY TESTS
Final Report

Barbara Fuller
Richard Thomas
James Konz
Lydia Thomas

October 1980

Supported by
US Army Medical Research and Development Command
Fort Detrick, Frederick, Maryland 21701

Contract No. DAMD17-78-C-8068

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SECURITY CLASSIFICATION OF THIS PAGE *(When Data Entered)*

REPORT DOCUMENTATION PAGE

READ INSTRUCTIONS BEFORE COMPLETING FORM

1. REPORT NUMBER	2. GOVT ACCESSION NO. AD-A104 765
	3. RECIPIENT'S CATALOG NUMBER
4. TITLE *(and Subtitle)* EVALUATION OF SHORT-TERM BIOASSAYS TO PREDICT FUNCTIONAL IMPAIRMENT Selected Short-Term Renal Toxicity Tests	5. TYPE OF REPORT & PERIOD COVERED Final Report September 1978 — July 1980
	6. PERFORMING ORG. REPORT NUMBER MTR80W00234
7. AUTHOR(s) Barbara Fuller, Richard Thomas, James Konz, Lydia Thomas	8. CONTRACT OR GRANT NUMBER(s) DAMD 17-78-C-8068
9. PERFORMING ORGANIZATION NAME AND ADDRESS The MITRE Corporation 1820 Dolley Madison Boulevard McLean, Virginia 22102	10. PROGRAM ELEMENT, PROJECT, TASK AREA & WORK UNIT NUMBERS 61102A.3E16112BS04 00.046.
11. CONTROLLING OFFICE NAME AND ADDRESS U.S. Army Medical Research and Development Command Fort Detrick, Frederick, MD 21701	12. REPORT DATE October 1980
	13. NUMBER OF PAGES 193
14. MONITORING AGENCY NAME & ADDRESS(*if different from Controlling Office*) U.S. Army Medical Bioengineering Research and Development Laboratory Fort Detrick, Frederick, MD 21701	15. SECURITY CLASS. *(of this report)* Unclassified
	15a. DECLASSIFICATION/DOWNGRADING SCHEDULE

16. DISTRIBUTION STATEMENT *(of this Report)*

Approved for public release; distribution unlimited

17. DISTRIBUTION STATEMENT *(of the abstract entered in Block 20, if different from Report)*

18. SUPPLEMENTARY NOTES

19. KEY WORDS *(Continue on reverse side if necessary and identify by block number)*

Renal	Pathology
Kidney	Biochemistry
Glomerular function	In vitro techniques
Tubular function	Toxic chemicals
Hemodynamics	Testing techniques

20. ABSTRACT *(Continue on reverse side if necessary and identify by block number)*

The MITRE Corporation has been requested by the U.S. Army Medical Bioengineering Research and Development Laboratory to identify and evaluate short-term bioassays which have demonstrated ability to assess and predict impairment of the renal system resulting from exposure to chemicals. This document reviews the literature on test procedures for determining effects on the kidneys and other components of the renal system. The procedures are discussed in sections on morphology, glomerular function, tubular function, intrarenal hemodynamics

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and biochemistry. Criteria for evaluating the procedures are given. A two-tiered testing system is recommended for a chemical renal toxicity screening program.

Level I of the testing system consists of quick and inexpensive but sensitive tests for renal injury with morphologic examination by gross observation and light microscopy. Level II tests are more complex but provide more information on the extent and mechanism of damage. These include clearance measurements, analyses for enzymes released by cellular injury, and renal plasma flow. Studies of excised tissue by in vitro techniques and microscopic examination of tissues obtained at sacrifice are recommended.

This report is accompanied by a directory entitled Development of Renal Bioassays in Laboratory Animals: Directory of Institutions/Individuals, which presents the names of organizations and individuals involved in the development and/or utilization of tests applicable to the screening of toxic substances in the renal system. The directory provides information concerning specific measurements performed, test systems employed, compounds tested, requirements for anesthesia, and terminal nature of the tests.

EXECUTIVE SUMMARY

The Metrek Division of the MITRE Corporation, under contract to the United States Army Medical Bioengineering Research and Development Laboratory, is reviewing and recommending short-term tests for evaluating and predicting the functional and/or morphological impairment produced by toxic substances using animal test systems. This document presents information on the available tests for the renal system and recommends those tests which are suitable for use in a screening program.

Nephrotoxicity may be manifested as glomerular damage, interstitial nephritis, tubular damage, disturbances in renal blood flow or any combination of these. Measurements of renal damage have been grouped into five categories based primarily on structural, functional or biochemical changes. These categories include: (1) morphological damage indicators, (2) glomerular function tests, (3) tubular function tests, (4) measurements of renal hemodynamics, and (5) enzymatic damage indicators. A variety of tests have been developed to detect renal damage, and many of these are well developed and have a demonstrated ability to indicate damage produced by nephrotoxins. Such tests are of particular interest in developing a screening program for nephrotoxicity, and are discussed in detail in this report.

Renal morphological abnormalities following exposure to toxic substances have been documented in humans and experimental animals.

The techniques used to measure damage range from gross observation to electron microscopy. Of the techniques employed, gross observation appears to be the least reliable since it is difficult to determine the significance of alterations in color, size or kidney weight unless combined with studies of renal function. Structural abnormalities in tissue and at the cellular and subcellular level can be determined by use of light- and electron microscopy. Light microscopy also lacks sensitivity in detecting damage, although it is an important technique in confirming damage in a screening program. Electron microscopy is a sensitive technique for detecting early damage and is useful in providing a thorough description of nephrotoxic damage to a system; however, it is more involved than light microscopy and is beyond the scope of most routine screening. It may have limited application in those studies where other tests provide inconclusive results.

Injury to the glomeruli may result from exposure to nephrotoxins. Several tests are available which may indicate glomerular dysfunction. These tests require only a blood or urine sample and are relatively simple to perform. Included among these tests are measurements of blood urea nitrogen (BUN), serum creatinine and protein in the urine. A variety of sensitive methods for measuring glomerular filtration rate (GFR) are also available when quantitative assessment of the degree of glomerular dysfunction is desired.

Measurement of serum creatinine is considered a more sensitive indicator of glomerular dysfunction than is BUN; however, neither test is suitable for the detection of early damage. Measurement of protein in the urine appears to be a sensitive indicator of early damage. The measurement of GFR using standard plasma clearance procedures is cumbersome and is not well suited for rapid screening. However, the plasma clearance procedures are the best developed methods for GFR determination and may be used in the later stages of a screening program. Plasma disappearance methods for determining GFR require further study but seem promising for screening.

The renal tubules are the main site of chemically induced nephroses. The main tests of tubular function are those which measure the reabsorptive and secretory functions and the ability of the kidney to concentrate the urine. In addition, general tubular damage can be detected by microscopic examination of the formed elements in the urine.

The reabsorptive function of the tubules can be assessed by measuring the levels of substances which are normally absent, or present in very low levels in the urine due to tubular reabsorption. The measurement of glucose in the urine, even though it is not a sensitive index of renal damage, is the more common test used in this regard.

Tests which measure the secretory function of the tubules include the measurement of urinary acidification, the measurement of

the transport maximum for p-aminohippurate and the in vitro evaluation of the renal transport of p-aminohippurate (PAH), N-methylnicotinamide (NMN) and tetraethylammonium ion (TEA). The urinary acidification test appears to be useful only for detecting severe damage. The measurement of transport maximum for PAH is useful for estimating the amount of active tubular mass. The in vitro techniques are useful indices of decreased tubular transport and have been shown to be sensitive indicators of tubular dysfunction. A measure of the ability of the kidney to concentrate the urine has also been shown to be a sensitive indicator of tubular dysfunction. The microscopic evaluation of urinary sediment is a valuable test of the anatomical integrity of the tubules.

Several procedures are available for measuring renal blood flow and intrarenal distribution of blood flow. The effective renal plasma flow can be determined by standard clearance procedures or by plasma disappearance methods. Regional blood flow and intrarenal distribution of blood flow may be determined through washout techniques using radioactive substances or through the extraction and entrapment of radioactive microspheres by the glomerular capillaries. Both the washout techniques and the microsphere technique provide valuable information concerning alterations in intrarenal hemodynamics.

Urinary enzyme determinations have been used as sensitive indicators of some forms of tubular damage. Experimental evidence has

shown that when tubular cells degenerate, the enzymes contained in the cells pass into the urine. Thus, changes in the urinary enzyme excretion rates may reflect alterations in tubular cells. A few urinary enzymes have been used in the screening of chemical substances for nephrotoxicity.

The renal system performs many functions related to glomerular filtration, tubular reabsorption and secretion and blood flow. Since the various renal functions are localized in specific sections of the renal system and the renal tests are generally applicable to only one type of renal function, a screening program should include a variety of tests capable of monitoring all of the various renal functions. A tiered screening program is recommended because the testing techniques can be subdivided into various levels of sophistication. For the renal system, the tests are subdivided into two separate levels.

Level I of the tiered program should consist of those tests which are simple, inexpensive, quick and sufficiently sensitive to provide a good indication of renal damage. Level I tests would include the measurements of glucose and protein. Additional urine tests would include specific gravity or osmolality, microscopic examination of urinary sediment and enzyme determinations.

Level II tests are more sensitive than Level I tests and should be better able to describe the extent and mechanisms of damage. These tests are also more time-consuming, more difficult to perform and more expensive. The tests included in this level include inulin

clearance, PAH clearance, PAH transport maximum, urinary acidification, in vitro cortical slices, ^{85}Kr washout, autoradiography, gross examination, and light- and electron microscopy. The assay of urinary enzyme activities is a sensitive technique for detecting some types of early renal tubular damage, and a few urinary enzymes would be useful in screening chemical substances for nephrotoxicity.

FOREWORD

The authors express their appreciation to Dr. Mary Henry, Project officer of the U.S. Army Medical Bioengineering Research and Development Laboratory, for the support and guidance that she provided during the course of the project. The expert contributions by William O. Berndt, Ph.D. and Donald E. Oken, M.D., who submitted critical reviews of this report in its draft form, is gratefully acknowledged, as are the contributions of Drs. Awadh Singh and Jean Weston. Leadership and advice by Dr. Paul Clifford throughout the course of the project have been of great value. The editorial and technical assistance by Ms. Lee Johnson and Ms. Yasuko Anglin, respectively, is sincerely appreciated.

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1.0 INTRODUCTION

The Metrek Division of The MITRE Corporation, under contract to the United States Army Medical Bioengineering Research and Development Laboratory, is reviewing and recommending short-term tests for evaluating and predicting the functional and/or morphological impairment produced by toxic substances using animal test systems. Effects in four organ systems--pulmonary, hepatic, renal, and cardiovascular--are being considered. This document presents information on the available tests for the renal system and recommends those tests which are suitable for use in a screening program.

Renal dysfunction can be produced by toxic agents, not only because of cytotoxic effects but also as a result of hemodynamic alterations. Nephrotoxicity may manifest itself as glomerular dysfunction, tubular dysfunction, disturbances in renal blood flow or any combination of these. A variety of tests have been developed to detect renal damage and many of these are well developed and have a demonstrated ability to indicate damage produced by nephrotoxins. Such tests are of particular interest in developing a screening program for nephrotoxicity and are discussed in detail in this report.

Measurements of renal damage have been grouped into the following five categories:

- Morphological Damage Indicators
- Glomerular Function Tests
- Tubular Function Tests

- Measurements of Renal Hemodynamics
- Enzymatic Indicators of Damage

This categorization is based primarily on the structural, functional, or biochemical changes that can be measured by the tests included within each category. Certain tests, particularly some of the simpler ones, may indicate damage to more than one component of the renal system (e.g., to glomerular and/or tubular function) and may best be described as tests of general renal function. In such cases, the test has been categorized according to the most probable type of damage reflected by an abnormal measurement. Morphological damage indicators (Section 2.0) include alterations in those structural characteristics that can be determined through gross observation or through the use of light- or electron microscopy. Tests of glomerular function (Section 3.0) include measurements which reflect abnormalities in glomerular filtration as well as in actual measurement of glomerular filtration rate. Assessment of tubular function (Section 4.0) involves evaluation of urinary concentrating and diluting ability and various in vivo and in vitro tests for tubular reabsorption and secretion. Examination of the urinary sediment for formed elements indicative of tubular injury has also been included in the Tubular Function category. Measurements of renal hemodynamics (Section 5.0) include measurements of effective renal plasma flow and intrarenal distribution of blood flow. Finally, Section 6.0

describes the measurement of urinary enzyme levels in detecting tubular damage.

Each test considered in this report is described in sufficient detail to give the reader an appreciation of its complexity and a familiarity with any special requirements for equipment, instrumentation or training. The significance of an abnormal test measurement is discussed in light of any confounding variables which may lead to false positive or negative values or otherwise affect the interpretation of test results. An attempt has been made to assess the extent to which each test is employed in investigations of renal dysfunction through qualitative estimates of the relative frequency with which the test is reported in the literature. For most tests, specific instances in which the test has been utilized to detect chemical nephrotoxicity in laboratory animals are presented.

The tests contained within each category vary widely in their sensitivity, accuracy, ease of performance and degree of sophistication. Each category therefore, contains tests suitable for various levels of a tiered testing program as well as tests which, for any number of reasons, may not be appropriate for inclusion in such a program. The suitability of each test for inclusion and probable placement in a testing scheme is mentioned in the discussion of that test; however, comparisons of tests within and among the various categories has been reserved for the final section of the report.

In Section 7.0, entitled Conclusions and Recommendations, criteria are defined for assessing the suitability of tests for inclusion in a nephrotoxicity screening program. Those tests considered in the report which satisfy these criteria are selected and classified according to a second set of criteria as belonging in Levels I or II of a tiered testing program. The tiered testing scheme that is presented is based upon a critical, comparative analysis of all of the renal tests currently used in small laboratory animals. This testing scheme, in the opinion of the authors, provides the most definitive information concerning the existence, nature and extent of chemically induced nephrotoxicity. Alternate or optional tests will be identified where appropriate.

Selected information concerning each test has been summarized in tabular form and is presented in appendices A through E. This information includes:

- the specific parameter measured,
- the species in which the test has been performed, and
- the substances that have been tested for toxicity or used to elicit a toxic response.

The "comments" column of each table contains information distilled from the body of the text pertinent to the suitability of the test for inclusion in a screening program.

In compiling a list of the animal species used for a particular test, only those in which the test has actually been performed are

mentioned. This information is presented in Table 1-1. This list does not imply that the test can only be performed in these species, but rather that use of the particular test has only been documented in certain animal models.

The information in this report has been assembled from published and unpublished literature, and communications with individuals active in the development or application of techniques for determining renal damage. Current activities of individuals and organizations involved in nephrotoxicity testing in animals have been compiled solely from personal communications and are presented in a companion directory to this report.

TABLE 1-1

SPECIES IN WHICH INDIVIDUAL INDICATORS OF RENAL FUNCTIONAL AND STRUCTURAL INTEGRITY HAVE BEEN DETERMINED

CATS	DOGS	GUINEA PIGS	HUMANS (CHILDREN)	HUMANS (ADULTS)	MICE	RABBITS	RATS	SWINE	SYRIEN GOLDEN HAMSTERS	
										MORPHOLOGICAL DAMAGE INDICATORS
X	X	X		X	X	X	X			GROSS OBSERVATION, TRANSMISSION ELECTRON MICROSCOPY (TEM), SCANNING ELECTRON MICROSCOPY (SEM)
										TESTS INDICATIVE OF GLOMERULAR DYSFUNCTION
	X			X	X		X			MEASUREMENT OF BLOOD UREA NITROGEN
X				X		X	X			MEASUREMENT OF SERUM CREATININE
X	X					X	X			MEASUREMENT OF PROTEIN IN THE URINE
										MEASUREMENT OF GLOMERULAR FILTRATION RATE: CLEARANCE PROCEDURES
	X		X	X		X	X	X		INULIN CLEARANCE
X	X		X	X		X	X	X		CREATININE CLEARANCE
	X		X	X			X			IOTHALAMATE AND DIATRIZOATE CLEARANCE
				X			X	X		UREA CLEARANCE
	X		X	X			X			OTHER SUBSTANCES
										MEASUREMENT OF GLOMERULAR FILTRATION RATE: PLASMA DISAPPEARANCE PROCEDURES
	X		X	X	X		X			IOTHALAMATE
				X						DIATRIZOATE
				X	X	X	X			**TUBULAR FUNCTION TESTS: REABSORPTIVE TESTS** MEASUREMENT OF GLUCOSE IN THE URINE
	X			X		X				MEASUREMENT OF TRANSPORT MAXIMUM FOR GLUCOSE
				X			X			**TUBULAR FUNCTION TESTS: SECRETIVE TESTS** MEASUREMENT OF URINARY ACIDIFICATION
	X			X		X	X			MEASUREMENT OF TRANSPORT MAXIMUM FOR PAH
X	X	X			X	X	X			IN VITRO RENAL CORTICAL SLICE TECHNIQUE
				X		X	X	X	X	**TUBULAR FUNCTION TESTS: URINARY CONCENTRATING AND DILUTING ABILITY** SPECIFIC GRAVITY AND OSMOLALITY
				X			X			**GENERAL TUBULAR DAMAGE:** MICROSCOPIC ANALYSIS OF URINE SEDIMENT
										MEASUREMENT OF RENAL HEMODYNAMICS: CLEARANCE PROCEDURES
	X		X	X		X	X			PARA-AMINOHIPPURIC ACID CLEARANCE
	X		X	X		X	X			ORTHOIODOHIPPURATE CLEARANCE
	X									IODOPYRACET CLEARANCE
										MEASUREMENT OF RENAL HEMODYNAMICS: DISAPPEARANCE PROCEDURES
	X		X	X			X			^{125}I or ^{131}I - ORTHOIODOHIPPURATE PLASMA DISAPPEARANCE
										MEASUREMENT OF INTRARENAL HEMODYNAMICS
	X			X			X			KRYPTON-85 AND XENON-133 WASHOUT
	X						X			RADIOACTIVE MICROSPHERE UPTAKE BY GLOMERULAR CAPILLARIES
										BIOCHEMICAL DAMAGE INDICATORS
X	X		X	X		X	X	X		URINARY ENZYME ACTIVITY

2.0 MORPHOLOGICAL DAMAGE INDICATORS

Renal morphological abnormalities following exposure to toxic substances (i.e., drugs, trace metals, organic solvents, pesticides) have been documented in humans and experimental animals by numerous investigators (Andrews, 1975; Dalhamn and Friberg, 1957; Fowler, 1972, 1974a, 1974b; Gritzka and Trump, 1968; Striker et al., 1968; Ware et al., 1973). The techniques used by these as well as other investigators range from gross observation of the intact organ (weight, size, color) to electron microscopy (ultrastructure). It should be noted that any technique used to determine morphological damage is complicated by the fact that the assessment is made by personal visual evaluation. Therefore, any morphological study must be preceded by a careful evaluation of the normal range of variation. Variation occurs among species and within species. The major factors that cause variation within species are age, sex and diet (Finn, 1977). The use of controls is the most satisfactory way to eliminate misinterpretation of normal variation in morphology. Although not a substitute for the use of controls in morphological screening tests, a number of histological studies of normal kidneys from small laboratory animals, particularly rats, have been conducted and are useful as background information (Rodin and Crowson, 1962a; Maunsbach, 1966; Rouiller and Muller, 1969).

The selection of the test species and strain is also critical to the use of a morphological test to indicate renal damage. The

importance of this aspect is made clear in a study conducted by Mazze et al. (1971). These investigators were attempting to find an animal model that could be used to demonstrate renal insufficiency due to metabolism of methoxyflurane. Adult male Fischer 344, Buffalo, Wistar, Sprague-Dawley, and Long-Evans rats were used. This study showed the Fischer 344 and Buffalo rats catabolized methoxyflurane to a greater extent than the other three strains. However, despite similar serum inorganic fluoride levels in the two strains, only the Fischer 344 developed renal histologic abnormalities.

2.1 Gross Observation and Light Microscopy

Of the techniques that could be employed, gross observation appears to be the least reliable. A number of studies refer to alterations in color, size or kidney weight following administration of a presumed nephrotoxin; however, there is difficulty in determining the significance of the change unless combined with studies of renal function. For example, in a study (Sharratt and Frazer, 1963) in which rats were administered uranyl nitrate (2 percent solution), the kidneys showed surface pitting and weight increases. At lower dose levels, however, little or no change is detected macroscopically, even though abnormal renal function could be correlated with administration of the nephrotoxin.

Normal rat kidneys may exhibit histologic features which might, in some instances, be mistaken for changes induced by nephrotoxic agents. These include hyaline casts and pyknotic nuclei in the loops

of Henle; epithelial detachment in the terminal segments of proximal convoluted tubules; focal cytoplasmic eosinophilia in tubular epithelium; disruption of cells along the track of knife nicks; and epithelial vacuolation in subcapsular tubules (Rodin and Crowson, 1962a).

Tubule cell injury is the most common lesion associated with nephrotoxins (Fowler, 1972; Gritzka and Trump, 1968; Rodin and Crowson, 1962a, 1962b; Taylor, 1965; Ware et al., 1973, 1975). A decrease in mitochondrial respiration or uncoupling of oxidative phosphorylation may contribute to tubular injury (Goyer, 1968). Fowler et al. (1975b), Kosek et al. (1974), and Mazze et al. (1973), have shown that some nephrotoxicants such as cadmium, gentamicin, and methoxyflurane produce dose-dependent damage in rats that can be observed using light microscopy.

2.2 Electron Microscopy

Both transmission (TEM) and scanning (SEM) electron microscopy have been used as techniques to describe renal damage at the cellular and subcellar level following administration of various toxicants. The level of resolution that can be accomplished with these instruments offers the investigator an increased ability to detect structural abnormalities, many of which could not be detected or adequately described using light microscopy. These include accumulation of lysosomes; lipid droplets and enlarged apical vacuoles; the absence of basal infoldings and brush border associated with the proximal convoluted tubules; detachment of the endothelial cells from

their basement membranes, and spherical microparticles embedded within the basement membrane; loss of ribosomes from surface membranes, and large smooth endoplasmic reticulum aggregates in the pars recta segment of the proximal tubules; thickening of the vessel walls in the renal arteries; and narrowing of the lumina by fibrocytic cell types. The obvious advantage is that the investigator is able to provide a far more extensive description of renal damage that may contribute to a better understanding of the mechanism(s) resulting in acute or chronic renal failure. Furthermore, some early structural changes related to damage can be detected using electron microscopy but not light microscopy. Even though it is more involved than light microscopy, a test for renal damage employing electron microscopy can be conducted within a short time period (from a matter of days to a few weeks), and since only small tissue samples are required, biopsies may be performed if large laboratory animals are used. Biopsies also offer the advantage of conducting additional tests since the animal would not be killed.

The major technical disadvantage is the same as that associated with light microscopy; namely, the evaluation is descriptive (qualitative) and the significance of the changes observed is subject to question. Indeed, some changes may have no functional significance. Also, in electron microscopy, only very small amounts of tissue are examined; thus information may not be obtained concerning the extensiveness of the changes.

In addition to the problems associated with the interpretation of results, unless a laboratory already has the equipment required to perform TEM or SEM studies, the initial investment can be large, depending upon the sophistication of the microscope purchased. All of the support equipment is also expensive, and highly trained laboratory technicians are required.

2.3 Summary

Based on the literature reviewed, it would appear that gross observation and light microscopic studies are not as sensitive as functional studies in detecting damage; nonetheless, they are an essential part of any pathologic examination procedure and would be appropriate in the later stages of a screening program. Electron microscopy is a sensitive technique for detecting early structural changes associated with nephrotoxicity and is important in thoroughly describing cellular damage, especially when the mechanisms of damage are being investigated. It is an involved procedure and is beyond the scope of a routine screening program; however, it may have limited application in the later stages of a program where the findings from other tests are inconclusive.

Morphological indicators of renal damage are summarized in Appendix A.

3.0 GLOMERULAR FUNCTION TESTS

Injury to the glomeruli may occur as a result of exposure to nephrotoxins, such as gentamicin and hydrocarbon solvents (Cohen et al., 1975; Finn, 1977). A screening program for renal damage should therefore include one or more tests for ascertaining impairment of glomerular function so that nephrotoxins, which primarily affect the glomeruli, do not go undetected. Several tests are available which may indicate glomerular dysfunction and are relatively simple to perform, requiring only a blood or urine sample. Included among these tests are measurement of blood urea nitrogen, serum creatinine and protein in the urine. While none of these tests are both specific for glomerular dysfunction and sensitive to early glomerular damage, all are quite useful as rapid screening procedures for nephrotoxicity and suggest glomerular involvement.

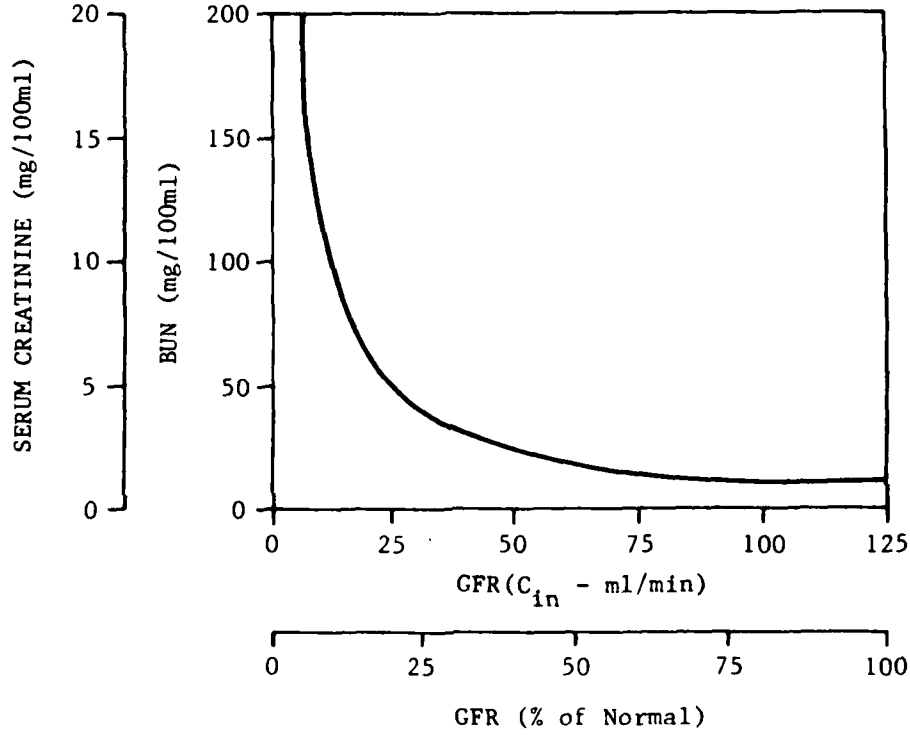
A variety of sensitive methods for measuring glomerular filtration rate are also available when quantitative assessment of the degree of glomerular dysfunction is desired. These procedures are more complicated to perform than the rapid screening tests mentioned above, and would therefore be suitable for later stages of a tiered screening program.

3.1 Rapid Nephrotoxicity Screening Tests Indicative of Glomerular Dysfunction

3.1.1 Measurement of Blood Urea Nitrogen

Urea, the primary end product of nitrogen metabolism in mammals, is excreted primarily as a result of glomerular filtration. When

filtration is impaired, plasma levels of urea rise in approximate proportion to the degree of impairment (Kassirer, 1971a). The blood urea concentration is usually expressed in terms of blood urea nitrogen (BUN). The relationship between glomerular filtration rate (GFR) and BUN is presented in Figure 3-1 along with the relationship between GFR and serum creatinine, another parameter used to assess glomerular function (See Section 3.1.2). While the values upon which this figure is based are for humans, a precise relationship between these parameters exists for any given species, and the BUN level provides an approximate index of the GFR as long as non-renal variables that alter BUN concentration remain stable (see below) (Berndt, 1976a; Crowe and Hatch, 1977). Since the concentrations of urea in the water of plasma and red cells are equivalent, BUN may be measured using either whole blood or plasma (Relman and Levinsky, 1971). Methods for measuring BUN require small quantities of blood (<0.2 ml) and are relatively simple. Manual determination is most frequently reported using the method of Gentzkow (1942), which involves conversion of urea to ammonia with urease and measurement of the ammonia by Nesslerization. Methods employing urease are not well suited for automated analysis since an incubation time of about 20 minutes is required for the conversion of urea to ammonia (Marsh et al., 1965). Autoanalytical techniques involving a direct reaction between diacetyl monoxime and urea are, however, available (Marsh et al., 1965) and have been used to determine BUN in dogs (Keogh et al., 1977) and rats (Cohen et al., 1975).



Modified from Berndt, 1976a

FIGURE 3-1
RELATIONSHIP OF GLOMERULAR FILTRATION RATE TO SERUM CREATININE CONCENTRATION AND BUN

Since the range of normal values for BUN is rather broad,* BUN is usually considered an unsatisfactory indicator of early renal damage involving limited impairment of glomerular function; values of BUN may remain within the normal range until glomerular function is substantially reduced (Wilson, 1975). Furthermore, an increase in BUN does not necessarily reflect decreased glomerular function, since tubular necrosis may result in a reduced clearance of urea (Carpenedo et al., 1974; Klein et al., 1972), and plasma urea concentrations are additionally affected by the rate of urine flow and nitrogen balance (Crowe and Hatch, 1977). In states of dehydration, BUN will rise while GFR remains normal. BUN will also rise in cases of accelerated protein degradation due to trauma, fever or infection; ingestion of large quantities of protein; and breakdown of blood in the gastrointestinal tract (Relman and Levinsky, 1971; Kassirer, 1971a; Crowe and Hatch, 1977).

Despite these disadvantages, the BUN is routinely used along with other tests in research laboratories to assess the effects of nephrotoxins in small animals (See Appendix B, Table B-1). If baseline values are obtained prior to exposure to the toxicant (using control animals or the animals to be tested), small increases in BUN may be more readily observed (Relman and Levinsky, 1971). In addition, the state of the animal with respect to diet, water intake,

*Values ranging from 10 to > 20 mg.% have been reported in various strains of rats (Oken, et al., 1966; Flamenbaum et al., 1971; Thiel et al., 1967; Klein et al., 1973); values ranging from 10 to 14 mg.% have been reported in dogs (Singhvi et al., 1978).

gastrointestinal bleeding and other factors which influence results of BUN determinations, can be controlled to a large extent. As a general screen for nephrotoxicity, BUN determinations are useful in detecting mild to severe dysfunction in experimental animals.

3.1.2 Measurement of Serum Creatinine

Creatinine is an end product of creatine metabolism in muscle, and its production and release from muscle is relatively constant (Kassirer, 1971a). After release, creatinine enters the plasma and is excreted by the kidneys exclusively via glomerular filtration in dogs, cats, and rabbits, and primarily via glomerular filtration (with additional tubular secretion) in rats and guinea pigs (Pitts, 1974). As is the case for urea, levels of serum creatinine vary inversely with GFR (see Figure 3-1). Unlike urea however, serum creatinine levels are influenced less by factors other than GFR, so that significant changes in the serum creatinine level are more indicative of alterations in glomerular function (Wilson, 1975).

Creatinine levels are easily measured in small quantities of plasma (0.2 ml.), and serum creatinine determinations have been performed successfully in experimental studies using rats and dogs (Martinez and Doolan, 1960; Cohen et al., 1975; Kaufman et al., 1977).*

*Normal values in rats have been reported as ranging from 0.4 to 1.2 mg.% (Cohen, et al., 1975; Carpenedo et al., 1974; Klein et al., 1973). Normal values in dogs have been reported as ranging from 0.9 to 1.3 mg.% (Kaufman et al., 1977).

Measurement of serum creatinine by the standard alkaline picrate method is a relatively simple colorimetric procedure (Martinez and Doolan, 1960); however, non-specific chromogens present in plasma react with the picrate reagent and render determinations obtained by this method somewhat inaccurate (Berndt, 1976a; Martinez and Doolan, 1960). Interference by non-creatinine chromogens and daily fluctuations in levels of serum creatinine (up to 10% [Crowe and Hatch, 1977]) may limit the utility of serum creatinine measurements; however, the proper use of baseline values and controls can alleviate some of the problems.

As a general screen for nephrotoxicity, serum creatinine determinations are useful in detecting mild to severe glomerular dysfunction in experimental animals. Serum creatinine is considered more sensitive in detecting renal dysfunction than is BUN; however, either determination would be useful in a short-term screening program (Wilson, 1975; Crowe and Hatch, 1977; Rickers et al., 1978).

3.1.3 Measurement of Protein in the Urine

The glomerulus is normally an effective barrier to the passage of most proteins from the plasma into the glomerular filtrate. Under normal conditions, proteins the size of serum albumin (M.W. approximately 70,000) and larger are retained, while smaller proteins (M.W. < 30,000) are filtered (Relman and Levinsky, 1971). Plasma proteins which pass through the glomerulus and into the filtrate are actively reabsorbed by the proximal tubules (Smith, 1960). Thus, protein is

normally present in only trace quantities in the urine of most mammals.

Proteinuria may be induced by nephrotoxic agents, which damage the glomeruli, permitting passage through the glomerular membrane of proteins that are normally retained; however, injury to the tubules, which results in decreased reabsorption of filtered proteins or leakage of protein from damaged cells, may also give rise to elevated concentrations of protein in the urine (Smith, 1960; Relman and Levinsky, 1971). Disorders of tubular transport in the absence of glomerular injury will generally result in increased quantities of low molecular weight proteins in the urine without significant albuminuria (Relman and Levinsky, 1971; Axelsson and Piscator, 1966).

In human clinical studies (Peterson et al., 1969; Hall, 1973), a low molecular weight beta$_2$-globulin (beta$_2$-microglobulin) was excreted in large amounts in patients with tubular dysfunction and only slight increases were observed in urinary albumin. In patients with glomerular disorders, normal to slight increases were observed in beta$_2$-microglobulin and large increases were observed in albumin excretion. No nephrotoxicity studies were found in the literature where specifically beta$_2$-microglobulin urinary clearance was examined in laboratory animals. Nonetheless, measurement of protein and albumin in the urine has been utilized to detect glomerular (as well as tubular) dysfunction following administration of a variety of nephrotoxins to small laboratory animals (see Appendix B, Table B-1).

The rat is the most frequently used species for such studies. Normal rats exhibit a slight proteinuria (Ellis et al., 1973b; Harman, 1971; Berndt, 1976a).* It is therefore necessary to obtain accurate control measurements, preferably in the individual experimental animals themselves, prior to administration of the nephrotoxin (Berndt, 1976a). Collection of a urine sample is usually accomplished through use of a standard metabolism cage and protein excretion is normally reported as mg./rat/24 hours or mg./100 ml. urine when quantitative analytical procedures are employed.

Several simple, semiquantitative procedures for measuring protein in the urine are available. One method depends upon the precipitation of protein by heat and acetic acid or by sulfosalicylic acid and comparison of the resulting turbidity with that of standards of known protein concentration. Precipitation methods are sensitive to concentrations of about 5 to 10 mg. protein/100 ml. (Relman and Levinsky, 1971; Wilson, 1975). The screening test for proteinuria most frequently used is the "dipstick" method, which involves alteration of an indicator dye by protein. The dye in common use is tetrabromphenol blue; however, the reaction is fairly specific for albumin and does not register the presence of other proteins (e.g. globulins) in the urine. The method is slightly less sensitive than the precipitation method, requiring albumin concentrations of 15-20 mg./100 ml.

*Values of 16.5 $\pm$ 1.9 and 9.2 $\pm$ 1.9 mg./24 hours have been reported for normal male and female rats, respectively (Harman, 1971).

(Relman and Levinsky, 1971). When greater accuracy is desired, total protein may be determined by the biuret reaction (Gornall et al., 1949). Very low concentrations of albumin can be accurately measured by radioimmunoassay techniques (Relman and Levinsky, 1971). An automated method for albumin determination using bromocresol green is also available (Beng and Lim, 1973).

Measurement of protein in the urine appears to be a sensitive indicator of glomerular damage in the rat. For example, a markedly increased urine albumin level was observed in rats following administration of nephrotoxic serum (Sharratt and Frazer, 1963). Microscopic examination revealed only mild glomerular damage. In another study, a severe proteinuria was observed in rats that were fed a diet containing N,N'-diacetylbenzidine for two weeks. The major component of the excreted protein was albumin. Once again, microscopic examination revealed lesions in approximately 30% of the glomeruli (Harman, 1971). It should be noted that in the latter study, glomerular lesions and accompanying proteinuria developed more slowly in male rats than in female rats; however, this seems to be an isolated occurrence and has not been observed in other studies (Oken, 1980). The proteinuria was eventually heavy in both male and female rats (Harman, 1971).

While occurrence of protein in the urine in excess of control values may indicate nephrotoxicity, proteinuria may also occur as a

result of damage to other organs, thus complicating the interpretation of results. For example, 24 hours following administration of paraquat to rats, excretion of protein increased almost fourfold over that of controls. Histologic examination, however, revealed only mild degeneration of the proximal tubules. Furthermore (and contrary to expectations in cases of tubular damage only), a high albumin to total protein ratio was observed (Lock and Ishmael, 1979). The severe proteinuria and increased excretion of albumin in this case does not reflect coexistent glomerular damage, but rather pulmonary injury. Albumin is released to the plasma from damaged lung tissue and, although the percentage of albumin filtered at the glomerulus (0.005%) remains constant, the quantity filtered is markedly increased.

In conclusion, measurement of protein in the urine appears to be a useful screening test for general nephrotoxicity. Additionally, the test may differentiate between tubular and glomular damage if albumin levels as well as total protein levels are determined, and if damage to other organs can be ruled out.

3.2 Measurement of Glomerular Filtration Rate

3.2.1 Plasma Clearance Methods

The glomerular filtration rate (GFR) is equivalent to the plasma clearance of a substance S provided that the substance: (1) is freely filterable at the glomerulus; (2) is neither secreted into, reabsorbed from nor synthesized by the tubules; (3) is not metabolized;

(4) is nontoxic and exhibits no pharmacologic effects; and (5) does not bind to plasma proteins.

Under these conditions, GFR may be measured using the standard clearance formula

$$GFR = C_s = \frac{[S]_u V}{[S]_p}$$

where C_s is the volume of plasma cleared of substance S per minute; $[S]_u$ is the concentration of S in the urine; V is the volume of urine exiting the ureter per minute and $[S]_p$ is the concentration of S in the plasma. A multiple series of determinations is performed and the values of GFR are averaged. Although a wide variety of substances have been utilized for measurement of GFR, only those which are commonly used and which give the most accurate results will be considered. These are discussed in Sections 3.2.1.1 through 3.2.1.3.

Measurement of GFR by clearance procedures requires maintenance of constant plasma levels of S and collection of accurately-timed blood and urine samples. Anesthesia is usually necessary, since collection of accurately-timed urine samples requires bladder catheterization, and maintenance of constant plasma levels of S may require constant intravenous infusion.

Clearance methods for determining GFR have been performed in standard laboratory animals, primarily in rats and dogs. Values ranging from 0.2 to 0.9 ml./min./100 g. have been reported in various strains of rat. (Gonick, et al., 1975; Barenberg et al., 1968;

Sharratt and Frazer, 1963; Flamenbaum et al., 1971; DiBona et al., 1971; Brennan et al., 1977). Values ranging from 2.9 to 4.0 ml./min./kg. have been reported in dogs (Singhvi et al., 1978; Pihl, 1974; Pihl and Nosslin, 1974). Intravenous infusion in rats is accomplished by femoral cut down; in dogs, catheterization of leg veins is used. Blood samples in rats are taken from the cut tail vein or from the retro-orbital plexus of the eye, sampling from the inner canthus with a capillary tube. Since vasoconstriction may cause stasis and lead to unreliable plasma values, the latter technique is preferred. In dogs, blood samples are taken from veins other than those receiving the infusion.

Changes in GFR, as measured by plasma clearance procedures, have been widely utilized as indicators of nephrotoxicity in laboratory animals (Appendix B, Table B-2). Most studies reported in the literature describe extensive damage following acute exposure to a toxicant. In these cases the depression in GFR has been severe (i.e., 50% or less of the control value). Further studies are needed to ascertain whether or not slight changes in GFR produced by a nephrotoxin can be detected using these methods.

3.2.1.1 Inulin Clearance. Inulin, a polyfructose of approximately 5,000 molecular weight, satisfies all of the criteria listed above. Inulin clearance provides an accurate measure of GFR in all mammals (Smith, 1960) and is commonly performed in rats and dogs. Since inulin is hydrolyzed to fructose in the gastrointestinal tract

and is poorly absorbed from subcutaneous tissue or muscle, it must be administered intravenously (Pitts, 1974). Maintenance of a constant plasma level requires infusion at a rate equivalent to the rate at which it is excreted. The chemical estimation of inulin is time-consuming and demanding (Relman and Levinsky, 1971; Pitts, 1974), although automated methods are available (Relman and Levinsky, 1971). Use of radioactive inulin, however, greatly simplifies the analytical procedure. Inulin containing ^{14}C or ^{3}H or allyl inulin (substitution of allyl ether groups for some of the OH groups) iodinated at the double bonds with ^{125}I, can be infused along with unlabeled carrier (Concannon et al., 1964; McCormack et al., 1978).

3.2.1.2 Creatinine Clearance. Creatinine satisfies the criteria for GFR determination via plasma clearance in dogs, cats and rabbits; however, creatinine is secreted by the tubules to a significant extent in rats and guinea pigs, and thus gives a high value for GFR as compared to inulin (Pitts, 1974). In dogs, creatinine clearance agrees to within $\pm$ 5% with inulin clearance (Pitts, 1974). While constant infusion is frequently employed, creatinine, unlike inulin, may be given subcutaneously (Smith, 1960). For accurate results, creatinine must be supplied in amounts sufficient to raise the plasma concentration to 15 mg./100 ml. or more. Although creatinine is normally present in the plasma at a concentration of approximately 1 mg./100 ml., clearance of endogenous creatinine is not usually considered a valid measure of GFR because of the non-specificity of the

standard alkaline picrate method of analysis. Noncreatinine chromogens, which react with the picrate reagent, are present in plasma but not in urine. Thus, the concentration of creatinine in plasma is falsely high, rendering the clearance of creatinine, as calculated by the standard clearance formula, lower than the actual GFR (Pitts, 1974; Sullivan, 1974). Utilizing a method for measuring "true" creatinine in plasma (Section 3.1.2), in combination with the standard picrate method for urinary creatinine analysis, may increase the utility of endogenous creatinine clearance for screening in dogs (Martinez and Doolan, 1960). Determining GFR in dogs by endogenous creatinine clearance has been reported in the literature using standard auto-analytical procedures (Keogh et al., 1977).

3.2.1.3 <u>^{125}I-iothalamate and ^{131}I-diatrizoate Clearance</u>. ^{125}I-iothalamate (IOT) clearance agrees well with inulin clearance and is commonly used to measure GFR in laboratory dogs and rats (Bryan et al., 1972; Oester et al., 1969). IOT may be administered by intravenous infusion; however, subcutaneous injection is also possible since the slow rate of release provides a blood level constant enough for accurate measurement of GFR (Wilson, 1975). ^{131}I-diatrizoate (DTZ) clearance has also been performed in dogs (Ram et al., 1969) and approximates inulin clearance. Estimation of GFR via DTZ clearance is not, however, reported frequently in the literature.

Aside from dispensing with the need for intravenous infusion, use of these compounds allows a simple and rapid analysis of plasma

and urine samples. However, caution should be exercised when DTZ and other iodinated substances are used in measuring GFR, because their clearance is species-dependent. For example, Mudge and coworkers (1971) compared the renal transport of DTZ in the rabbit, dog and rat, and found that the dog clears DTZ solely by glomerular filtration, but the rabbit actively secreted DTZ and the data on the rat were equivocal. For this reason, species differences should be established before studies using these substances are undertaken.

3.2.2 Plasma Disappearance Methods

The need for accurate urine collection renders conventional plasma clearance techniques for determining GFR (as discussed in Section 3.2.1) relatively cumbersome in small laboratory animals such as rats. Moreover, since the bladder or ureter must be catheterized, chronic experiments are frequently complicated by superimposed cystitis and pyelonephritis. In addition, when intravenous infusion is required, repeated clearances are difficult to perform, especially in the rat, due to the need for venous cutdowns (Blaufox et al., 1967a). These problems may be circumvented by the use of plasma disappearance methods, which do not require urine collection. While there is little doubt that the standard clearance technique is superior to any other technique for determining GFR in terms of accuracy and reliability, plasma disappearance methods have also proven to be quite accurate and reliable (Truniger et al., 1968; Silkalns et al., 1973; Pihl, 1974; Powers, et al., 1977). In these methods, a substance(s),

which is removed from the body principally by glomerular filtration, is administered in a single intravenous dose. This is accomplished easily in the rat through the tail vein or the dorsal vein of the penis. A leg vein is suitable in dogs. A series of timed blood samples is taken and the concentration of S remaining per unit volume of plasma is determined and plotted vs. time on semilog paper. Radioactively-labeled substances such as ^{125}I-IOT and ^{131}I-DTZ are commonly employed for these determinations so that concentration is expressed in CPM/ml. (Figure 3-2).

GFR is most simply calculated using the following formula:

$$GFR = V\beta$$

where V = the volume of distribution and β = the rate of decline of plasma concentration. V is obtained by dividing the CPM administered by the CPM/ml. at zero time, which in turn is determined by extrapolating the "slow phase" of the plasma disappearance curve to zero (Figure 3-2) as given by the following formula:

$$= \frac{\log_e 2}{t^{1/2}} = \frac{0.693}{t^{1/2}}$$

where $t^{1/2}$ is the half-life of the disappearance curve.

More complicated mathematical analyses which reportedly increase the accuracy of the calculations have been employed (Stokes and Ter-Pogossian, 1964; Farmer et al., 1967; Powers et al., 1977); however, determinations of GFR which agree well with those obtained via

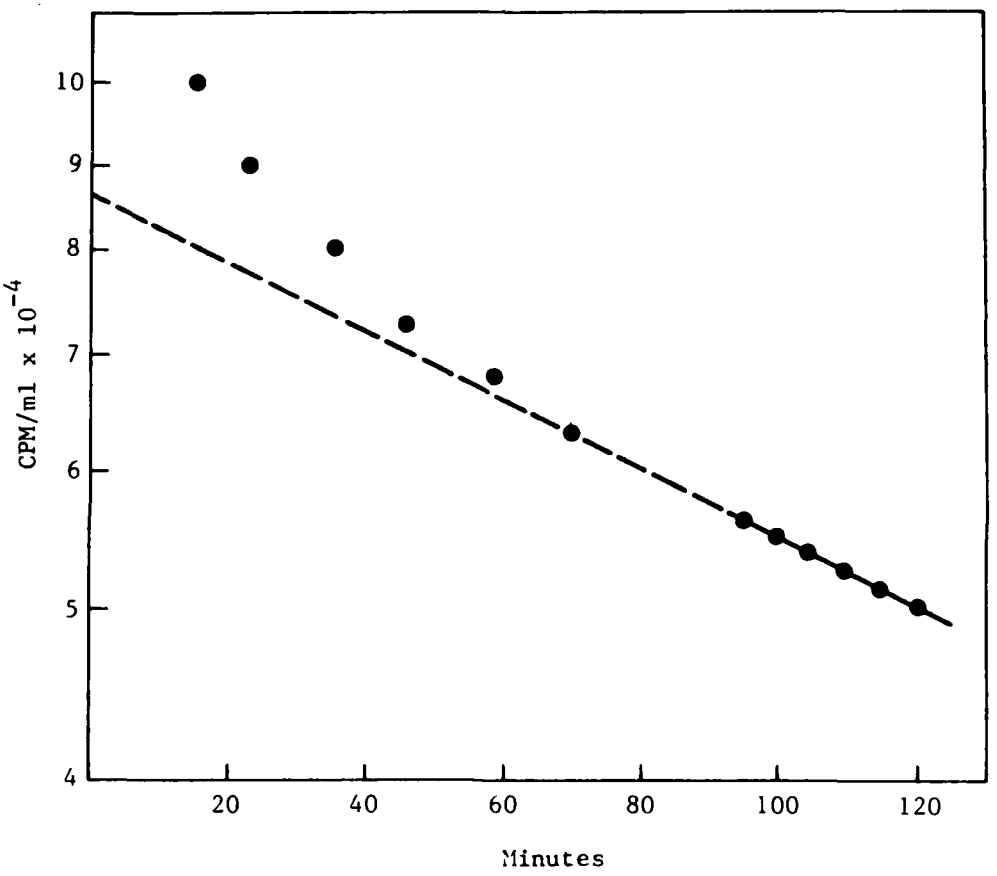


FIGURE 3-2
THE DISAPPEARANCE CURVE PLOTTED ON SEMILOGARITHMIC PAPER

inulin clearance, or clearance of S by standard procedures, have been reported using this simple approach (Ram et al., 1969; Bryan et al., 1972).

Plasma disappearance methods seem promising for screening since they provide a simple, rapid, accurate and easily repeated measure of GFR in small animals. In some instances, these procedures have been performed in the absence of anesthesia (rats [Bryan et al., 1972] and dogs [Powers et al., 1977]).

3.3 Summary

Tests which may indicate glomerular dysfunction include measurement of blood urea nitrogen, serum creatinine and protein in the urine. In addition, several methods for quantitative determination of the glomerular filtration rate are available.

Measurement of BUN, serum creatinine and protein in the urine can be performed on standard laboratory animals such as rats and dogs, require small volumes of blood or urine and involve simple analytical techniques. Since blood urea levels are affected by a variety of factors such as rate of urine flow and nitrogen balance and may increase in cases of tubular necrosis, the serum creatinine level is generally considered a better indicator of glomerular dysfunction than is the BUN. Neither test is suitable for detecting early damage since a small increase in either parameter may result in a value still within the normal range. Both tests are, however, valuable for rapid nephrotoxicity screening.

Proteinuria may occur as a result of either glomerular or tubular injury, or may accompany damage to other organs such as the lung. Massive proteinuria (especially excretion of excessive quantities of albumin) is usually considered to be indicative of glomerular damage when damage to other organs can be ruled out.

Since severe proteinuria has been observed in rats in cases of mild glomerular injury (as judged by histological examination), measurement of protein in the urine appears to be a sensitive indicator of early damage in this species. The sensitivity of this test does, however, vary with the sex, being greater in females for certain nephrotoxins.

The glomerular filtration rate may be measured by standard plasma clearance procedures employing inulin, creatinine and ^{125}I-iotholamate (IOT) or ^{131}I-diatrizoate (DTZ). All clearance procedures require collection of timed urine samples (as well as timed blood samples) and thus bladder catheterization. Inulin clearance additionally requires constant intravenous infusion. These methods are cumbersome and are therefore suitable only for later stages of a tiered screening program when accurate determination of GFR may be desired.

Plasma disappearance methods for determining GFR using ^{125}I-IOT or ^{131}I-DTZ seem promising for screening since they only involve a single intravenous injection and collection of timed blood

samples. GFR, as measured by disappearance methods, agrees well with values obtained by plasma clearance methods.

While acute depression of GFR can be easily measured using either clearance or disappearance methods, further studies are needed to ascertain whether or not small changes in GFR accompanying mild glomerular injury can be detected.

Glomerular function tests are summarized in Appendix B.

4.0 TUBULAR FUNCTION TESTS

The renal tubules are the main site of chemically-induced nephrosis (Balazs et al., 1963). Because of this, tests which measure tubular function are of great importance in a short-term screening program for nephrotoxicity. Unlike the glomerulus which has one function, the tubules carry out many functions. It is therefore necessary to have several tests to measure these various tubular functions since no single test would be adequate.

The main tests of tubular function are those which measure the reabsorptive and secretory functions and the ability of the kidney to concentrate the urine. In addition, general tubular damage can be detected by microscopic examination of the formed elements in the urine. The following sections discuss the tubular function tests, which may be useful in a small animal nephrotoxicity screening program.

4.1 Reabsorptive Tests

The plasma contains many substances of low molecular weight which are readily filtered at the glomerulus, but are normally absent or present at very low levels in the urine. These substances are reabsorbed by the tubules before they can be excreted in the urine. Some of the substances that are reabsorbed at the tubules include the following: carbohydrates, amino acids, acetoacetic acid, lactic acid, uric acid, beta-hydroxybutyric acid, vitamins; and important ions such as phosphate, sulphate, bicarbonate, sodium, potassium and

chloride (Pitts, 1974). Most of the reabsorption occurs within the proximal tubule segment. These substances are reabsorbed by active transport mechanisms against a concentration gradient, and in some cases, a gradient of electrical potential. The transport mechanisms are thought to involve specific enzyme systems. Nevertheless, the reabsorptive processes are limited to maximal rates for each substance absorbed.

Glucose is one of the substances actively transported in the tubules. Decreases in the transport rates of glucose may be indicative of damage to the active transport system for this substance. The following sections discuss the techniques used to measure impairment of glucose reabsorption. These sections also describe the usefulness of these techniques in detecting damage in the tubules of the kidneys.

4.1.1 Measurement of Glucose in the Urine. Glucose is almost completely reabsorbed by the proximal tubules (Berndt, 1976a) and normally does not appear in the urine except in trace amounts. Its presence in the urine above trace quantities is suggestive of tubular dysfunction (Balazs et al., 1963; Crowe and Hatch, 1977; Diezi and Biollaz, 1979; Kassirer, 1971b); however, glucosuria cannot be considered a specific index of tubular damage, since a disturbance in carbohydrate metabolism (e.g., diabetes), as well as other abnormalities, can also result in the appearance of glucose in the urine (Guyton, 1976). These other causes must be considered when

glucosuria is encountered before assuming that tubular dysfunction exists. It is common practice, for example, to compare the plasma glucose level with the urinary glucose level to show that the glucosuria is independent of hyperglycemia (Wilson, 1975).

The resorptive capacity of the tubules for glucose is greatly diminished following nephrotoxic insult. As a consequence, glucose is excreted into the urine in moderate to large amounts (Berndt, 1976a). For example, glucose has been detected in the urine of rabbits following administration of uranyl nitrate (Nomiyama et al., 1974) and of rats treated with uranyl nitrate and mercuric chloride (Balazs et al., 1963). In the dose-response study with rabbits, Nomiyama et al. (1974) administered a single intravenous injection of uranyl nitrate at a dose of either 0, 0.1, 0.2, 0.5 or 1.0 mg uranium/kg body weight. The rabbits were then evaluated for renal damage 24 and 48 hours after injection. Urinary glucose levels were found to be significantly increased in rabbits receiving doses of 0.2 mg/kg and greater. This test was not as sensitive an indicator as the urinary enzyme assay used in this study, and other investigators have reported that glucosuria does not occur in the early stages of damage in the proximal convoluted tubules (Balazs et al., 1963). However, the test for glucose is relatively simple and can give a rough indication of renal damage.

Glucose in the urine can be determined both quantitatively and semiquantitatively; however, a quantitative measurement is not

considered essential in assessing renal disease (Wilson, 1975). Glucose can be analysed in the urine semiquantitatively by "dipstick" methods. In this procedure, the test strip is dipped into the urine and alterations of the indicator dye are compared with a standard color chart (Balazs et al., 1963; Plaa and Larson, 1965). Other analytical techniques to estimate the glucose concentration in blood and urine are available; the most common is the glucose oxidase test. A major problem with this test is the false negative reaction that can occur in the presence of large amounts of ascorbic acid (Wilson, 1975).

It does not appear that the measurement of glucose in urine is as sensitive an index of renal damage as other available tests (Diezi and Biollaz, 1979; Nomiyama et al., 1974). However, this test is suitable as a screening test at Level I of a tiered testing system for nephrotoxicity in small animals.

4.1.2 Measurement of the Transport Maximum for Glucose

As plasma concentrations of glucose are increased, a point is reached where tubular reabsorption attains a constant, maximal rate (glucose transport maximum, Tm_G). Once saturation is attained, all glucose in excess of Tm_G is excreted in the urine. The transport maximum for glucose is determined by loading the tubular cells with more substance than can be reabsorbed. To determine Tm_G, an intravenous dose of glucose is given to raise the plasma level and then a high plasma level is maintained with a constant infusion of glucose.

The bladder of the animals is drained, using a catheter, and then urine samples are collected for specified collection periods, using an indwelling catheter (Vander, 1963). Throughout the collection period, blood samples are taken. At least two different plasma concentrations must be utilized in the beginning of the study to be assured that tubular saturation is indeed present, even though glucosuria would tend to indicate saturation. The concentration of glucose in the blood and urine samples is then determined using the analytical methods described in Section 4.1.1. The Tm_G is calculated as follows:

$$Tm_G = ([G]_p \times GFR) - ([G]_u \times V)$$

Where $[G]_p$ = the concentration of glucose in the plasma; [G]u = the concentration of glucose in the urine; GFR = the glomerular filtration rate and V = the volume of urine excreted per unit time.

To determine Tm_G, the glomerular filtration rate (GFR) must first be determined (See Section 3.2). Tm_G has only been used to a limited extent to study the renal effects of toxic substances (Miller et al., 1950; Nomiyama and Foulkes, 1968; Vander, 1963). Nomiyama and Foulkes (1968) noted inhibition in the tubular capacity to reabsorb glucose following uranyl acetate poisoning in rabbits. Vander (1963), in studying the effects of the salts of zinc, cadmium and mercury on renal transport systems, found inhibition of glucose reabsorption and a decrease in the glucose transport maxima for animals

exposed to either zinc or mercury. He found no changes in Tm_G for animals treated at a concentration of 200 μ/kg. cadmium.

Many abnormalties (e.g., diabetes, hyperthyroidism, liver disease) can lead to glucose in the urine and thus distort the values calculated for Tm_G (Krupp and Chatton, 1979). Even when such abnormalties can be ruled out, measurement of Tm_G does not appear to provide enough information over and above that obtained through simple measurement of glucose in urine to justify the greater complexity of this test. Therefore, the measurement of Tm_G would appear to have only limited application in a screening program.

4.2 Secretory Tests

Several substances are secreted into the proximal, distal and collecting tubules of the kidneys. These include hydrogen ions, potassium ions, urate ions and some synthetic organic acids and bases such as p-aminohippurate (PAH) and N-methyl-nicotinamide (NMN) Hydrogen ions may be secreted in combination with the reabsorption of buffers such as bicarbonate, phosphate and ammonium ions. Tubular acidosis indicates damage to the proton secretory mechanisms of the tubules.

PAH and NMN are used to determine the tubular capacity for secretion. For example, the quantity of PAH secreted in the proximal tubules is proportional to the tubular mass with active transport mechanisms for this substance. When the active transport mechanisms are damaged there is a decrease in the rate of secretion.

Nonetheless, the secretive processes are limited to maximal rates for each substance. This probably occurs because one step of the secretory process becomes saturated during elevated concentrations of either organic acids or bases.

The maximal tubular secretion rates for these substances can be determined experimentally. Decreases in the secretion rates can provide an indication of decreased capacity due to damage in the tubules. The following sections describe the techniques used to measure impairment of hydrogen ion, organic acid and organic base secretion. These sections also describe the usefulness of these techniques in detecting damage in the tubules of the kidneys.

4.2.1 Measurement of Urinary Acidification

Hydrogen ions, which are evolved during metabolic processes, are excreted into the proximal, distal and collecting tubules. The secretion of these ions into the proximal tubule results in the reabsorption of bicarbonate, a process essential for maintaining hydrogen ion equilibrium. When hydrogen ion secretion into the proximal tubules is affected, bicarbonate reabsorption is less efficient and bicarbonaturia occurs (Brenner and Rector, 1976; Diezi and Biollaz, 1979). When the damage is to the distal tubules, hydrogen ion transport is affected, resulting in an inability to acidify the urine to minimal levels. The result of either of these dysfunctions is a condition known as renal tubular acidosis (Diezi and Biollaz, 1979; Kassirer, 1971b; Morris, 1969; Rodriguez-Soriano and Edelmann, 1969).

The kidney regulates acid-base balance by regulating the concentration of plasma bicarbonate (Morris, 1969). The pH of the urine is a measure of the ability of the kidney to respond to disturbances in the acid-base balance. The most common test of acidification in animals measures the minimal urinary pH after stimulation of proton secretion by loading with various compounds (e.g., phosphate, ammonium chloride). Normally this procedure results in a urinary pH in rats of between 5 and 5.6 (Diezi and Biollaz, 1979).

Several investigators have studied the effects of nephrotoxins on the urinary acidification process in rats (Edwards et al., 1971; Gouge and Andriole, 1971; Rector, 1973). In the study by Gouge and Andriole (1971), amphatericin B (a known nephrotoxin) was administered to rats for 21 days. An ammonium chloride solution was then administered for three days to stimulate proton secretion. Urine was collected on the third day and the pH was determined using a pH meter. The ability of the kidney to acidify the urine was found to be greatly diminished. Control animals were able to produce a minimal urinary pH of 4.86, while the minimal pH for treated animals was 6.06. The authors concluded that the defect in acidification pointed to distal tubular degeneration. The proximal tubules were considered normal since there was no observed glycosuria or proteinuria.

The test for urinary acidification has been used as a screening test to detect renal tubular acidosis in humans; however, since it is not a sensitive index of kidney dysfunction, it is not considered

adequate alone and must be used with complementary tests (Rodriques-Soriano and Edelmann, 1969). It appears that this test is useful for detecting severe renal damage (i.e., renal tubular acidosis); however, no conclusions regarding the utility of this test for detection of early renal dysfunction were found in the literature.

4.2.2 Measurement of Transport Maximum for PAH

The transport maximum of p-aminohippurate (TM_{PAH}) is occasionally used to estimate the amount of active renal tubular mass in the kidneys and can be used as an indication of functional damage in the proximal tubules where PAH is secreted. The transport maximum for PAH is determined by loading the tubular cells with more substance than can be secreted. The load is the total amount of PAH in the plasma that passes through the kidney each minute. For instance, if the concentration of PAH in the plasma is 20 mg/100 ml and 5 ml of plasma passes through the kidneys each minute, then the PAH load is 1 mg/minute. To measure Tm_{PAH}, an intravenous dose of PAH is given to raise the plasma level to the desired concentration. This level is maintained with a constant infusion of PAH to replace that which is excreted.

The bladders of the animals are emptied by use of a catheter or by light suprapubic pressure, and then the animals are either placed in metabolism cages for urine collection or the urine is collected using an indwelling catheter. During the collection period, blood samples are also taken. The PAH plasma level is then increased and

new urine and blood samples are collected. The concentration of PAH in the blood and urine are then determined using the analytical methods described in Section 5.1.1.1. The transport of PAH is calculated as follows:

$$T_{PAH} = ([PAH]_u \times V) - ([PAH]_p \times GFR)$$

where T_{PAH} is the rate of secretion of PAH in mg./min. at each of two $[PAH]_p$; $[PAH]_u$ = the concentration of PAH in the urine; $[PAH]_p$ = the concentration of PAH in the plasma; GFR = the glomerular filtration rate; and V = the volume of urine excreted per unit time.

When the T_{PAH} does not increase from one collection period to the next, even though the plasma level of PAH has increased, the secretory transport mechanisms for PAH are saturated, and Tm_{PAH} has been attained. To determine Tm_{PAH}, the glomerular filtration rate (GFR) must be determined (See Section 3.2).

Tm_{PAH} has been determined principally in rats (Sharratt and Frazer, 1963), rabbits (Nomiyama and Foulkes, 1968; Nomiyama et al., 1973), and dogs (Singhvi et al., 1978; Vander, 1962, 1963). Singhvi et al. (1978) administered single intravenous doses of 0.5 mg. of uranyl nitrate/kg. body weight to dogs and found reversible renal impairment and marked decreases in Tm_{PAH}. Similarly, in earlier studies by Nomiyama and Foulkes (1968), rabbits administered uranyl acetate by intravenous injection had high levels of inhibition of tubular function and significant decreases in Tm_{PAH}. In studies in

Vander (1963), cadmium and zinc salts administered to dogs inhibited PAH transport and reduced the transport maxima for PAH. Sharratt and Frazer (1963) found that the mean control value in rats for Tm_{PAH} was 0.824 $\pm$ 0.028 mg./min. During their studies, they found that the Tm_{PAH} values were laborious to determine with nonradioactive analytical techniques; that they required considerable experience to obtain reproducible results; and that the values varied from one researcher to another and among different strains of rats. The more recent techniques (Lock, 1979) use radiolabeled PAH (e.g., P-amino-[^{3}H] hippurate) which simplifies the analytical procedures for determining PAH in both plasma and urine.

Even though Tm_{PAH} is used to estimate the level of tubular damage, it seems to be insensitive to early stages of damage (Diezi and Biollaz, 1979; Sharratt and Frazer, 1963). Tm_{PAH} is also altered by various factors, such as extracellular fluid volume expansion and uneven damage in different nephrons (Brickner and Schultze, 1972), and it may be altered without evidence of renal histological damage (Sharratt and Frazer, 1963).

Tm_{PAH} is cumbersome to determine even with the more recent radioactive alytical techniques, is insensitive to early renal damage, and can be influenced by external factors. Consequently, this test would have limited application in early routine nephrotoxicity screening. However, Tm_{PAH} measurements may be useful in the later

stages of a tiered screening program for estimates of active renal tubular mass.

Organic bases such as tetraethylammonium ion (TEA) and N-methylnicotinamide (NMN) are actively secreted by the tubules, and their transport maxima may be determined by the same methods used to determine Tm_{PAH}. Such measurements could provide estimates of active renal tubular mass and indicate functional damage. However, TEA and NMN are primarily used in in vitro studies of tubular transport (See Section 4.2.3) and have had only limited application in in vivo studies, where they were used for clearance determinations and not for the determinations of transport maxima (Lock, 1979).

4.2.3 In Vitro Evaluation of the Renal Transport of PAH, NMN and TEA Using Cortical Slices and Isolated Tubules

The in vitro renal cortical slice technique can be used to evaluate functional and biochemical phenomena in the renal system (Berndt, 1976b). Inhibition of PAH accumulation, as well as accumulation of NMN or TEA by renal cortical slices, can serve as sensitive indicators of nephrotoxicity (Berndt, 1976a, 1976b; Chow et al., 1977; Hirsch, 1973a, 1973b, 1976; Watrous and Plaa, 1972a, 1972b). This in vitro inhibition of organic acid and base accumulation corresponds to decreased tubular transport.

Renal cortical slices may be prepared from excised kidneys after the administration of a nephrotoxin to the animals. Two methods are used to prepare renal cortex tissue slices. The first method involves free-hand slicing of the kidney tissue and has the advantage

of being a quick preparation procedure. The disadvantage is that the slices lack uniform thickness. The second method is slower than the free-hand procedure; however, slices can be obtained that have uniform thickness. The second method involves the use of the Stadie-Riggs microtome, which mechanically slices tissues from a tissue cube with a constant pressure cutting blade (Burg and Orloff, 1973). After the slices are cut, they are incubated for 90 minutes under 100% oxygen in an isotonic medium containing either PAH, ^{14}C-PAH, ^{14}C-NMN or ^{14}C-TEA. Then PAH content of the renal slices and the medium are analyzed colormetrically, while the ^{14}C-labeled compound concentrations are determined, using a liquid scintillation counter. The data are expressed as the ratio of the renal slice concentration (mmoles of substance/g. of tissue) to the medium concentration (mmoles/ml.) (S/M). If this ratio exceeds one, it is indicative of an active transport process, and if the value is one or less, it is indicative of damage to the active processes, or for some other substances, it may indicate passive transport.

Berndt (1976b) has reported that PAH uptake by renal cortical slices can serve as the counterpart model for the in vivo tubular secretory process. Furthermore, Berndt (1976a) and Hirsch (1976) suggested that the in vitro renal cortical slice technique may be more sensitive than in vivo methods for determining nephrotoxic effects on renal transport processes because blood flow effects are eliminated. Hirsch (1976) also indicated that the in vitro renal

cortical slice technique is routinely used to determine the effect of substances on renal organic acid and base transport.

Many substances such as uranyl nitrate, potassium dichromate, mercuric chloride, lead, gentamicin, halogenated hydrocarbons and ochratoxin A have been tested in different species of animals (e.g., mice, rats, rabbits and dogs) using this technique, and the results indicated the toxicity of these substances by inhibiting accumulation of PAH, NMN or TEA in the renal cortical slices (Berndt, 1976c; Hirsch, 1972, 1973a, 1973b, 1974; Hirsch et al., 1971; Hook et al., 1974; Stroo and Hook, 1977a, 1977b; Suzuki et al., 1975; Watrous and Plaa, 1972a, 1972b). Watrous and Plaa (1972a, 1972b) found that the renal cortical slice technique is a sensitive procedure for determining the nephrotoxicity of some chlorinated hydrocarbons in mice. Chow et al. (1977) observed a dose-related depression of PAH and NMN accumulation in renal cortical slices prepared from triclosan- or chlorhexidine-treated rats. Several other investigators (Berndt, 1976c; Berndt and Hayes, 1977; Stroo and Hook, 1977a) have found dose-related responses in small laboratory animals with various nephrotoxins using this in vitro technique.

Berndt (1976a, 1976b) and Hirsch (1976) suggested that the in vitro renal cortical slice technique is extremely useful in assessing acute renal toxicity; however, care must be taken to ensure that renal tissue slices are properly prepared and examined. The renal cortical slice technique should be useful in a screening program to

assess nephrotoxicity. The advantages of this technique include the following: the chemical composition of the ambient fluid can be fairly rigidly controlled; the influence of certain external factors that may alter tubular excretion in the intact animal can be easily controlled; the technique permits examination of various metabolic inhibitors that cannot be tolerated in live animals; and the simplicity of the technique enables a greater number of observations than can be obtained from intact animals. The major disadvantage is that it requires that the animals be terminated.

The in vitro perfusion of isolated tubule segments may be used to examine transport in the renal tubules. Perfusion and sample collection are performed through pipettes attached to each end of the tubule segment. The perfusion medium is passed through the perfusing pipette into the tubular segment and then collected in the collecting pipette. The rate of perfusion flow in most studies is between 2 and 24 ml/minute (Burg and Orloff, 1973). During perfusion, the segments are suspended in a bath which is bubbled with the same gas mixture as the perfusion media. In tubular secretion studies, the substance secreted is added to the bath media. In absorption studies, the substance absorbed is added to the perfusion media. Following perfusion, the concentrations are determined in the bath and the perfusion media (Tune et al., 1969). The most common cortical slice tubules used are from rabbits because they are easily removed. Dissection of the tubules is difficult, if not impossible in dogs, rats, mice,

guinea pigs, hamsters, frogs, toads and Necturi. The only other animal from which kidney tubules have been removed is the flounder (Burg and Orloff, 1973). The following segments have been successfully perfused: proximal convoluted tubule, proximal straight tubule, descending limb of Henle's loop, thick ascending limb and the cortical collecting tubule.

Tune et al. (1969) studied PAH transport using the perfusion of isolated rabbit proximal tubules. They found active PAH secretion in both the convoluted and the straight portion of the proximal tubules, and that PAH was actively transported into the tubule cell at the peritubular membrane and subsequently diffused into the luminal fluid.

One of the advantages of perfused tubules is that it is possible to correlate flux measurements and cell composition in single experiments. Intracellular solute concentration, transport rates and permeabilities of the renal tubule can be measured in isolated tubules by the use of radioisotopes. An additional advantage of the in vitro perfusion technique is that individual segments of the nephron can be examined.

The in vitro renal slice technique is very useful in detecting renal damage and it is a sensitive technique for evaluating nephrotoxic effects on the renal transport process. It should be a useful method in a short-term screening program. The perfused renal tubule technique can be used to examine transepithelial transport of

substances such as PAH and NMN; although principally a research technqiue, it is suitable for Level II screening.

4.3 Urinary Concentrating Ability

The ability of the kidney to concentrate urine depends on a complex mechanism involving tubular transport of various substances, tubular responsiveness to antidiuretic hormone, and renal medullary blood flow (Crowe and Hatch, 1977). While a decrease in glomerular filtration may impair urinary concentrating ability, alterations in urinary concentration are generally considered primarily to reflect tubular integrity, and such abnormalities often develop when GFR is within the normal range (Crowe and Hatch, 1977; Relman and Levinsky, 1971; Wilson, 1975). Loss of ability to concentrate urine occurs not only when the distal tubules are damaged but in cases of proximal tubular damage as well. Measurements of urinary concentrating ability are therefore somewhat nonspecific, in that the precise mechanism of injury frequently remains unknown (Berndt, 1976a; Crowe and Hatch, 1977; Diezi and Biollaz, 1979). A depressed urinary concentrating ability is, however, characteristic of a wide variety of renal disorders (Berndt, 1976a; Diezi and Biollaz, 1979), including those resulting from exposure to nephrotoxins, such as chromium (Berndt, 1976a) and uranyl nitrate (Sharratt and Frazer, 1963). Furthermore, this effect is observed early in the development of renal impairment (Berndt, 1976a; Crowe and Hatch, 1977). Since measurements of concentrating ability are additionally simple and convenient to perform,

they are the most widely used methods for estimating tubular function (Relman and Levinsky, 1971).

The two methods which are used to determine the concentrating ability of the kidney are the measurement of urine-specific gravity and the measurement of urine osmolality. Specific gravity is the ratio of the weight of a volume of urine to the weight of the same volume of distilled water. Thus, specific gravity of urine depends both on the number of solute molecules and their density (Wilson, 1975).

Osmolality is defined as the number of solute particles per unit of solvent. This test is preferred over specific gravity since it more closely measures factors which pertain to the physiology of the concentrating process, and is generally considered to be the more accurate of the two measurements (Crowe and Hatch, 1977; Relman and Levinsky, 1971; Wilson, 1975).

Most studies to detect abnormalities in concentrating ability involve measurement of maximum concentrating ability. These studies require that fluid be withheld for a specified period of time prior to urine collection. The specific gravity or osmolality can then be determined using various techniques.

The specific gravity of a urine sample can be measured using one of several instruments, depending on the volume of urine available. A hydrometer can be used when 25 ml. or more of urine are available (Relman and Levinsky, 1971; Street, 1970). When smaller amounts of

urine are available, a pycnometer, with an approximate capacity of 0.5 ml., can be utilized (Sharratt and Frazer, 1963). A light refractometer, which can measure urinary concentration directly, can be used in place of a pycnometer or a hydrometer. The use of the refractometer is practical for even very small amounts (a single drop) of urine and has been found useful, practical and reliable even in mice (Balazs et al., 1963; Street, 1970).

Measurements of osmolality are based on the principle of freezing point depression and are obtained directly from an osmometer. When a freezing-point depression apparatus is available, the measurement of osmolality is easy, rapid (30 seconds per sample) and quite precise. In addition, only small volumes of urine are required (50 to 75 μl), rendering the test suitable for studies in small animals such as rats (Diezi and Biollaz, 1979). However, osmometers are more expensive than the instruments utilized for measurement of specific gravity. Thus, while the results of specific gravity and osmolality tests are not precisely interchangeable, osmolality measurements are not sufficiently superior to specific gravity measurements to warrant widespread substitution, and either test will provide a useful first approximation of the concentrating ability of the kidney.

Many factors can affect urinary specific gravity. The values can be elevated in certain diseases (e.g., fever, diarrhea, vomiting, exudation from burns) and diminished in others, especially those

associated with a high volume of urinary output (e.g., chronic interstitial nephritis, acute renal insufficiency). Even substantially decreased fluid intake will elevate, and increased intake lower, the urine-specific gravity (Relman and Levinsky, 1971; Wilson, 1975). The specific gravity can also be affected by certain drugs, dyes, refrigeration of the urine sample, proteinuria and glycosuria (Relman and Levinsky, 1971).

Although many of the factors which affect specific gravity (e.g., glocosuria, proteinuria) do not appreciably affect osmolality (Crowe and Hatch, 1977), there are extrarenal factors which must be considered. For example, systemic hypertension or a low protein diet can decrease urine osmolality (Foulkes and Hammond, 1975). The osmolality may also be depressed under the influence of various drugs, such as corticosteroids and diuretics (Wilson, 1975).

Measurements of urinary specific gravity and osmololity have been performed in many species of laboratory animals and have been utilized to detect renal damage following administration of nephrotoxins. The measurements are usually made on urine from animals deprived of water overnight. The approximate normal range of specific gravity for all species is 1.015 to 1.050 (Siegmund and Frazer, 1973). In the dog, normal specific gravity values should be 1.040 or above. Values between 1.035 and 1.040 indicate the possibility of renal tubular damage, while values consistently below 1.035 are indicative of renal damage. Because rats normally have a slight

proteinuria, normal values and limiting ranges cannot be given; however, specific gravity may be found according to the following formula (Street, 1970):

True SG = SG measured - (mg. protein/100 ml. x 0.003).

Diezi and Biollaz (1979) have reported the normal value for maximal urinary osmolality in female rats to be approximately 2448±57 mOsm/L. This maximal concentration resulted following 48 hours of water deprivation.

Sharratt and Frazer (1963) included specific gravity measurements in a battery of tests to determine the sensitivity of various measurements for detecting tubular damage in water-deprived rats resulting from exposure to known nephrotoxins. These investigators used a pycnometer, with a capacity of approximately 0.5 ml., to measure the specific gravity of rat urine following experimental renal damage. An abnormal specific-gravity measurement (significantly different from the 1.059 reading of the control group) occurred in four out of five groups with acute tubular damage and six out of nine groups with chronic tubular damage. None of the groups with glomerular damage were found to have abnormal specific-gravity readings. Only the measurement of cells in the urine was a more sensitive test of tubular function in these studies.

Berndt (1975) utilized osmolality measurements to detect tubular damage resulting from exposure to potassium dichromate. Potassium dichromate was administered to rats in single doses of 10 mg./kg. or

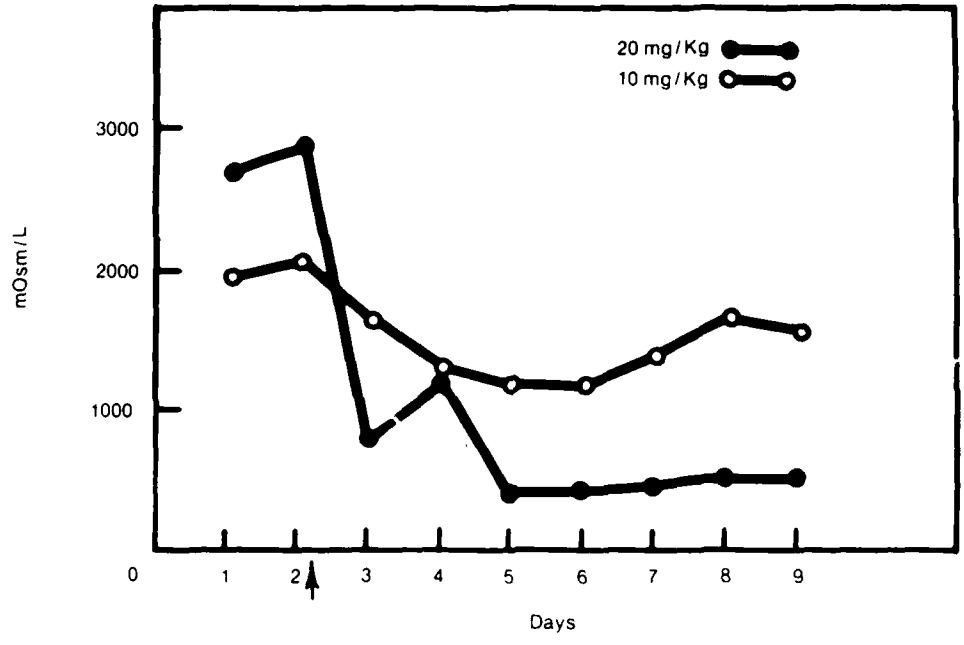
20 mg./kg. Control urine samples were collected for two days prior to nephrotoxin administration. Osmolality was monitored for several days thereafter. Figure 4-1 shows the result of this study. As can be seen, the normal ability of the rats to concentrate the urine was lost after the administration of chromium. A very dilute urine was produced for the remainder of the experiment.

The urine concentration test can best be characterized as the simplest, most reliable first approximation technique presently available to provide a relatively dependable measure of tubular function (Crowe and Hatch, 1977; Relman and Levinsky, 1971; Sharratt and Frazer, 1963; Wilson, 1975). It deserves to be utilized in any small animal screening study where nephrotoxic effects are to be determined.

4.4 Diluting Ability

Dilution of the urine is a tubular function which can be affected independently of the concentrating function. Dilution tests are much less useful, and a far less critical gauge of renal function than are concentrating tests; and they are affected by factors other than those relating to the kidney's concentrating ability (Relman and Levinsky, 1971).

Sharratt and Frazer (1963) performed this test on rats by giving them 5% of their body weight of tap water by gastric intubation. The urine was collected at 30-minute intervals for 120 minutes, and the volume and specific gravity of each sample determined.



Source: Adapted from Berndt (1975)

FIGURE 4-1
CHANGES IN URINARY OSMOLALITY FOLLOWING SUBCUTANEOUS ADMINISTRATION OF TWO DOSES OF POTASSIUM DICHROMATE

The volume of urine passed in 120 minutes was expressed as the percentage of water given. Two out of four groups, treated with nephrotoxic agents to produce acute tubular damage, showed abnormal readings when compared with controls in both volume and specific-gravity measures.

Determination of urinary diluting ability is of limited value in assessing renal tubular damage. An abnormality in this renal function occurs relatively late in the course of renal disease and can be affected by many nonrenal disorders such as congestive heart failure, adrenal insufficiency, hepatic disease and inappropriate secretion of antidiuretic hormone (Crowe and Hatch, 1977). This test has not been used extensively. Sharratt and Frazer (1963) found that the diluting ability was less sensitive than the concentrating test in detecting renal damage.

4.5 General Tubular Damage

The microscopic evaluation of urinary sediment is an important part of any evaluation of kidney damage. Although it provides no information about renal function, it is a valuable test of anatomical integrity of the kidneys (Relman and Levinsky, 1971).

The urine sediment consists of epithelial cells, leukocytes, erythrocytes, casts and crystals. In normal urine, small numbers of epithetial cells are present. These cells derive not only from desquamation from the tubular walls, but also from other portions of the urinary tract. However, in all types of renal disease, including

damage resulting from experimental nephrotoxic agents, the tubular epithelium degenerates, resulting in the appearance of increased numbers of epithelial cells in the urine.

The excretion of large numbers of relatively well-preserved, free renal epithelial cells, and casts containing such cells, is usually a sign of an active tubular degenerative process. In acute tubular necrosis, plaques of renal epithelial cells may appear in the urine (Relman and Levinsky, 1971).

Although leukocytes and erythrocytes appear in normal urine in small numbers, it is not clear how or where they enter urine. It is not conclusive that they derive from the kidney. In renal disease, however, increased numbers of red and white cells appear in the urine. Since these cells may be derived from the kidney, as well as other parts of the urinary tract below the kidney, they must be seen in casts, which are formed in the tubules, before it can be certain that they derive from the kidney (Relman and Levinsky 1971).

Casts are typically cylindrical masses of agglutinated material which are formed in the distal parts of the nephron and are then washed out into the urine. There are two main processes which appear to be responsible for the formation of casts--the agglutination of masses of cells in the tubular lumina and/or the intratubular precipitation or gelling of protein present in tubular fluid. Usually there are not enough cells in the tubular lumina and not enough protein in distal parts to form casts. The excretion of increased

numbers of casts usually means either increased proteinuria and/or renal excretion of cells.

The size of casts is determined by the dimensions of the tubule in which they are formed. The broadest casts are believed to be formed in the collecting tubules and the ducts of Bellini. The presence of many broad casts in the urine suggests widespread stasis of urine and cessation of excretion in large segments of the kidney and is therefore indicative of advanced renal disease (Relman and Levinsky, 1971).

Crystals appear in the urine as a result of several factors. These include the amount of various compounds in the urine, the pH of the urine, the volume of urine, and tubular reabsorption functions. Small numbers of crystals (e.g., uric acid, carbonates, oxalates, phosphates) normally appear in the urine (Wilson, 1975). Increased numbers of crystals appearing in the urine could indicate renal damage, especially if other signs of damage are present (e.g., appearance of increased numbers of cells or casts). This analysis requires that the pH and temperature of the urine be controlled, since crystals may precipitate out as the sample cools (Wilson, 1975).

Several investigators have discussed and/or used this technique in small animal studies (Balazs et al., 1963; Diezi and Biollaz, 1979; Lock and Ishmael, 1979; Prescott and Ansari, 1969; Sharratt and Frazer, 1963). Prescott and Ansari (1969) studied the effects of mercuric chloride administration on exfoliation of renal tubular

cells in rats. At dose levels sufficient to produce tubular necrosis, a striking increase in the number of cells appearing in the urine was observed. The number of cells in the urine appeared to be dose-related and the observed latency period between the start of treatment and the rise in tubular cell counts was inversely proportional to the dose.

This test is simple, routine and easy to perform. The sediment is examined using ordinary bright-field, light microscopy; however, this examination should be done by an experienced technologist (Relman and Levinsky, 1971; Street, 1970; Wilson, 1975). It is usually sufficient to make a qualitative assessment of the types and relative numbers of the elements in the urine sediment.

It would appear that a determination of the types and numbers of cells, casts and crystals in the urine provides data useful for screening purposes. Such studies could readily determine areas of the tubule which have been injured, since typical cells from the different sections can be readily differentiated.

4.6 Summary

Tests which measure tubular function are an important part of a screening program for nephrotoxicity since the tubules are the site of most chemically-induced nephroses. The most common tests are those which measure the reabsorptive and secretory functions. In addition, tests of urinary concentrating and diluting ability, and tests which measure general tubular damage, are also available.

The reabsorptive function of the tubules can be assessed by measuring the levels of substances which are normally absent, or present in very low levels in the urine due to tubular reabsorption. Glucose is one such substance. Following nephrotoxic insult, the mechanisms for transporting glucose are affected. As a result, the resorptive capacity of tubules for glucose may be greatly diminished and glucose is excreted into the urine. Its presence in urine strongly suggests tubular dysfunction, once other possible causes have been ruled out. However, since many other factors can be responsible for glucosuria, this test is not as sensitive or specific as other available tests.

Another test which measures the maximal rate of glucose reabsorption (the glucose transport maximum test [TM_G]) has also been used to a limited degree. This test is more complex and does not appear to provide enough additional information to justify its use over that of simple urinary glucose measurement.

Tests which measure the secretory function of the tubules include the measurement of urinary acidification, the measurement of the transport maximum for PAH and the <u>in vitro</u> evaluation of the renal transport of PAH, NMN and TEA.

The urinary acidification process is affected following damage to either the proximal or distal tubules. When hydrogen ion secretion into the proximal tubules is affected, bicarbonate reabsorption is less efficient and urinary pH increases. When distal tubules are

damaged, hydrogen ion transport is affected, resulting in an inability to acidify the urine. By monitoring urinary pH, the tubular dysfunction can be determined. However, this test appears to be useful only for detecting severe renal damage.

The transport maximum for PAH is a secretive test which can be used to estimate the amount of active tubular mass and can indicate functional damage in proximal tubules, where PAH is secreted. Since the quantity of PAH secreted into the proximal tubules is proportional to the tubular mass, the rate of secretion is decreased when the tubules are damaged. This test can measure the decrease in the secretory rate.

The in vitro renal cortical slice technique can also be used to evaluate tubular function. The test measures the inhibition of accumulation of PAH, NMN and TEA by the tissue slices. This inhibition has been shown to correspond to decreased tubular transport of these substances. This technique has been reported to be very useful and sensitive for evaluating nephrotoxic effects on the renal transport process. It has many advantages over in vivo techniques which measure the same functions; however, the animals must be terminated.

The in vitro perfusion of isolated tubule segments is another technique which may be used to determine tubular transport. This is principally a research technique, however, and is not considered suitable for routine screening.

A measure of the ability of the kidney to concentrate the urine has been shown to be a sensitive indicator of tubular dysfunction. Loss of the ability to concentrate urine occurs following damage to either the proximal or distal tubules. The two methods used to determine the concentrating ability are the measurement of specific gravity and osmolality. Either method can be used; however, specific gravity is used more routinely. The test of urine concentrating ability has been characterized as the simplest, most reliable first approximation technique presently available to provide a relatively dependable measure of tubular function.

Determination of urinary diluting ability has also been used to evaluate tubular damage. This test appears to be of limited value, since abnormalties in this function occur late in the course of renal disease and can be affected by many nonrenal disorders.

General tubular damage can be determined by the microscopic evaluation of urinary sediment. Although this provides no information about renal function, it is a valuable test of anatomical integrity, since an increase in the number of urinary cells and casts is indicative of tubular degeneration. A qualitative assessment of the types and relative numbers of elements in the urine is simple, routine and easy to perform. In addition, it is possible to determine which areas of the tubules have been injured, since cells from different tubules can be differentiated. It would appear that this test could provide data useful for screening purposes.

Tubular function tests are summarized in Appendix C.

5.0 MEASUREMENTS OF RENAL HEMODYNAMICS

A basic function of the kidneys is to clean the plasma of unwanted substances. The ability to carry out this function depends to a large extent upon the availability of an adequate blood supply. Under normal conditions, approximately 20 to 25% of the cardiac output flows through the kidneys so that the kidneys are supplied with more blood per gram of tissue weight than any other organ in the body (Finn, 1977). Alterations in either the absolute amount of blood flowing through the kidneys or the intrarenal distribution of blood flow can be expected to have a substantial impact upon renal function. The effects of toxic agents on the regulatory processes within the kidney which act to minimize changes in blood flow that might accompany alterations in arterial blood pressure, circulating blood volume or peripheral vascular resistance, are largely unknown. A reduction in outer cortical blood flow, with a redistribution of blood flow to the inner cortex has, however, been suggested to result from exposure to nephrotoxins (Finn, 1977). Tubular necrosis may also accompany a reduction in renal blood flow following exposure to a nephrotoxic substance, such as the antitumor agent inosine dialdehyde (Kaufman et al., 1977).

Several procedures are available for measuring renal blood flow and intrarenal distribution of blood flow. While none of these procedures are suitable for inclusion in Level I of a tiered screening program for nephrotoxicity, many are well developed and routinely

utilized in research laboratories for the assessment of renal damage (see Appendix D).

Procedures for measuring renal blood flow are rarely employed as the sole indicator of nephrotoxicity, but are usually performed in conjunction with measurement of glomerular filtration rate or as part of a battery of renal function tests. The sensitivity of renal blood-flow measurements for detection of early renal damage, especially in laboratory animals such as the rat (Churchill et al., 1977), cannot be ascertained from the available literature; however, some studies suggest that alterations in glomerular or tubular function may be observed prior to alterations of renal blood flow. For example, chronic exposure of dogs to polymixin antibiotics resulted in depression of glomerular filtration rate and maximum tubular function, but not of renal plasma flow in those animals exhibiting moderate damage. Only in animals in which damage was severe was renal blood flow also depressed (Moyer et al., 1953).

5.1 Measurement of Renal Blood Flow

5.1.1 Plasma Clearance Methods

The quantity of a substance S entering the kidney per unit time via the renal artery may be defined as

$$RPF \times [S]ap$$

where RPF is the renal plasma flow and [S]ap is the concentration of S in the arterial plasma, ap. Similarly, the quantity of a substance leaving the kidney is

$$RPF \times [S]vp + [S]uV$$

where [S]vp is the concentration of S in the venous plasma, vp, [S]u is the concentration of S in the urine and V is the volume of urine excreted in the given time (Sullivan, 1974).

If S is not synthesized, stored or destroyed, by the renal parenchyma, then the quantity S entering the kidney is equal to the quantity of S leaving the kidney. Thus

$$RPF \times [S]ap = RPF \times [S]vp + [S]uV.$$

Solving for RPF we get

$$RPF = \frac{[S]uV}{([S]ap-[S]vp)}.$$

The fraction of S removed from the plasma during a single passage through the kidney is known as the extraction ratio, E, and has a maximum value of 1. In cases where S is completely filterable at the glomerulus and all the S remaining in the plasma following glomerular filtration is secreted into the tubules, E will approach 1. Under these conditions, no S will appear in the venous plasma and [S]vp will equal 0. Thus

$$RPF = \frac{[S]uV}{[S]ap} = C_s.$$

Several substances are available which have extraction ratios approaching 1. For these substances, the plasma clearance approximates the renal plasma flow and RPF may be determined in the laboratory through standard clearance procedures similar to those used for determination of GFR (Section 3.2.1). The RPF so determined is believed to represent the plasma perfusing the functional tubular

tissue, rather than the total RPF, and is known as the effective renal plasma flow (ERPF) (Smith, 1960). The true renal plasma flow may be determined from the ERPF provided that E is known:

$$RPF = \frac{ERPF}{E} = \frac{C_S}{E.}$$

The renal blood flow may then be calculated as follows:

$$RBF = \frac{RPF}{1 - \text{hematocrit.}}$$

Clearance procedures for measuring ERBF have many of the same requirements and disadvantages as those for measuring GFR. Maintaining constant plasma levels of S and collecting accurately-timed urine samples usually involve constant infusion and bladder catheterization respectively, and anesthesia is normally required. As was discussed in Section 3.2.1, bladder catheterization may lead to infection. Intravenous infusion in rats requires venous cutdowns, rendering repeated measurements difficult to perform (Blaufox et al., 1967b). In addition, since processes which damage the kidney (e.g., nephrotoxins) may markedly alter the extraction ratio, E, accurate determinations of RPF involve simultaneous determination of E in the experimental animal (Sullivan, 1974). Determining E requires sampling arterial blood and renal venous blood. In dogs and rats, arterial blood is usually obtained from the femoral artery, although use of the carotid artery has been reported in rats (Johnson and Kleinman, 1979). Arterial blood may be obtained from rabbits via the central artery of the ear (Sadowski et al., 1977). Renal venous blood may

be obtained in all species through catheterization of the renal vein.

The substances most commonly used in clearance procedures for determining ERPF are para-aminohippuric acid (PAH) and orthoiodohippurate (Hippuran). Iodopyracet (Diodrast) is also used, but less frequently.

5.1.1.1 PAH Clearance. The clearance of PAH is the classical procedure for measuring ERPF and is the standard by which all other procedures are judged. PAH is completely filterable at the glomerulus, and the quantity remaining in the plasma after filtration is largely removed by tubular secretion when [PAH]ap does not exceed the tubular transport maximum (Sullivan, 1974). The exact value for E varies from species to species and within a given species, but is generally within the range of 0.75 to 0.90 in rats and dogs (Blaufox et al., 1967; Gyrd-Hansen, 1968; Johnson and Kleinman, 1979).

Values of ERPF in rats, as determined by PAH, have been reported to range between 1.88 and 2.82 ml./min./100g. (Blaufox et al., 1967b; Brennan et al., 1977). Values in dogs range from 9.05 to 15.77 ml./min./kg. (Powers et al., 1977; Stokes and Ter-Pogossian, 1964). Lower values were obtained in dogs anesthetized with sodium pentobarbital than in dogs anesthetized with methoxyflurane. The highest values were obtained when tests were performed in unanesthetized animals (Powers et al., 1977).

The standard analytical technique for measuring PAH is the diazotization method of Bratton and Marshall (1939), as modified by Smith et al., (1945). This colorimetric procedure, while still used to some extent, is generally considered to be tedious and time-consuming, and has largely been replaced by methods which employ ^{14}C or ^{3}H-PAH (Brennan et al., 1977; McCormack et al., 1978).

5.1.1.2 Clearance of Iodohippurate and Iodopyracet. Orthoiodohippurate (OIH) and Iodopyracet, like PAH, are completely filterable at the glomerulus, effectively secreted by the tubules and have values of E approaching 1 (Smith, 1960). These substances are therefore suitable for measuring ERPF by clearance techniques and have been utilized for this purpose for many years (Tauxe and Hunt, 1966).

OIH may be labelled with either ^{131}I or ^{125}I allowing simple, rapid and accurate analysis of blood and urine samples by scintillation counting (Tauxe and Hunt, 1966). Substitution of labelled OIH for PAH was originally intended to simplify the analytical procedures during evaluation of renal function in clinical studies; however, this method has also been used in experimental determinations of ERPF, particularly in dogs (Pritchard et al., 1965; Summers et al., 1967).

When ^{131}I labeled IOH is employed, care must be taken to remove free ^{131}I from the tracer. O ^{131}IH has been found to be contaminated to variable degrees with free ^{131}I and, if the latter

is not removed prior to infusion, values for C_{OIH} will be lower than values for C_{PAH} (Cutler and Glatte, 1965; Summers et al., 1967). This problem may be avoided by using ^{125}I labelled OIH. When either o ^{125}IH or ^{131}I-free o ^{131}IH is utilized for determination of ERPF by clearance procedures the values obtained correlate well with those obtained from C_{PAH} (Cutler and Glatte, 1965; Summers et al., 1967).

5.1.2 Plasma Disappearance Methods

ERPF may be determined by measuring the disappearance of a suitable material from the plasma following a single intravenous injection. The procedures are essentially identical to the plasma-disappearance methods used for determining GFR, and are described in Section 3.2.2.

A substance utilized for determining ERPF by plasma disappearance methods must satisfy the same criteria as one utilized for determining ERPF by plasma clearance methods. That is, the substance must be completely filterable at the glomerulus, secreted by the tubules and not synthesized by the renal parenchyma. OIH satisties these criteria and either ^{125}I or ^{131}I labelled OIH is the substance most commonly used for this test (Gott et al., 1962).

The dog is the most popular animal for renal hemodynamic studies, and determining ERPF by plasma disappearance methods (as well as by plasma clearance methods) is most frequently reported in the literature in this animal (Binnion and Cumming, 1967; Kaufman

et al., 1977; Pihl, 1974; Powers et al., 1977; Pritchard et al., 1965; Razzak et al., 1965; Stokes and Ter-Pogossian, 1964; Summers et al., 1967). While some disagreement exists regarding the accuracy of ERPF measurements as determined by plasma disappearance methods (Pihl and Nosslin, 1974), excellent agreement with values obtained by PAH or OIH clearance procedures has been observed by numerous investigators (Cutler and Glatte, 1965; Gott et al., 1962; Pihl, 1974; Pritchard et al., 1965; Stokes and Ter-Pogossian, 1964; Summers et al., 1967; Tauxe and Hunt, 1966).

The advantages of plasma disappearance methods for determining ERPF are similar to those described in Section 3.2.2 for use of these methods in determining GFR. The need for bladder catheterization and intravenous infusion is eliminated, and the procedure can be performed without anesthesia. However, these methods are somewhat less suitable for screening than similar methods for measuring GRF since accurate measurement of ERPF ultimately requires determining E, which in turn requires renal vein catheterization (Pritchard, 1965).

5.2 Measurement of Regional Blood Flow and Intrarenal Distribution of Blood Flow

Standard clearance procedures or disappearance methods for determining renal plasma flow provide information regarding overall blood flow through the kidney, but are of little value in determining regional blood flow or distribution of blood flow through the various layers of renal tissue. Several methods are available for measuring intrarenal hemodynamics; two of these are well developed and commonly

used for assessing renal function in laboratory animals. The commonly used methods are:

(a) measurement of the washout of Krypton-85 (^{85}Kr) and Xenon-133 (^{133}Xe) from the kidney, and

(b) measurement of the extraction and entrapment of radioactive microspheres by the glomerular capillaries.

5.2.1 Washout Techniques

In the standard washout method for measuring regional blood flow, ^{85}Kr is dissolved in saline and rapidly injected into the renal artery through a catheter. The rate at which this radioactive material is washed out of the kidney is measured by an appropriately positioned external scintillation detector (Pitts, 1974). Measurement of blood flow by this method is based upon the assumption that a highly diffusible substance will equilibrate between blood and tissue in a single passage through the capillaries. The rate at which the substance is removed will therefore vary directly with the rate of blood flow through the capillaries, and if the tissue/blood partition coefficient is known, the blood flow per unit volume of tissue can be determined (Thorburn et al., 1963).

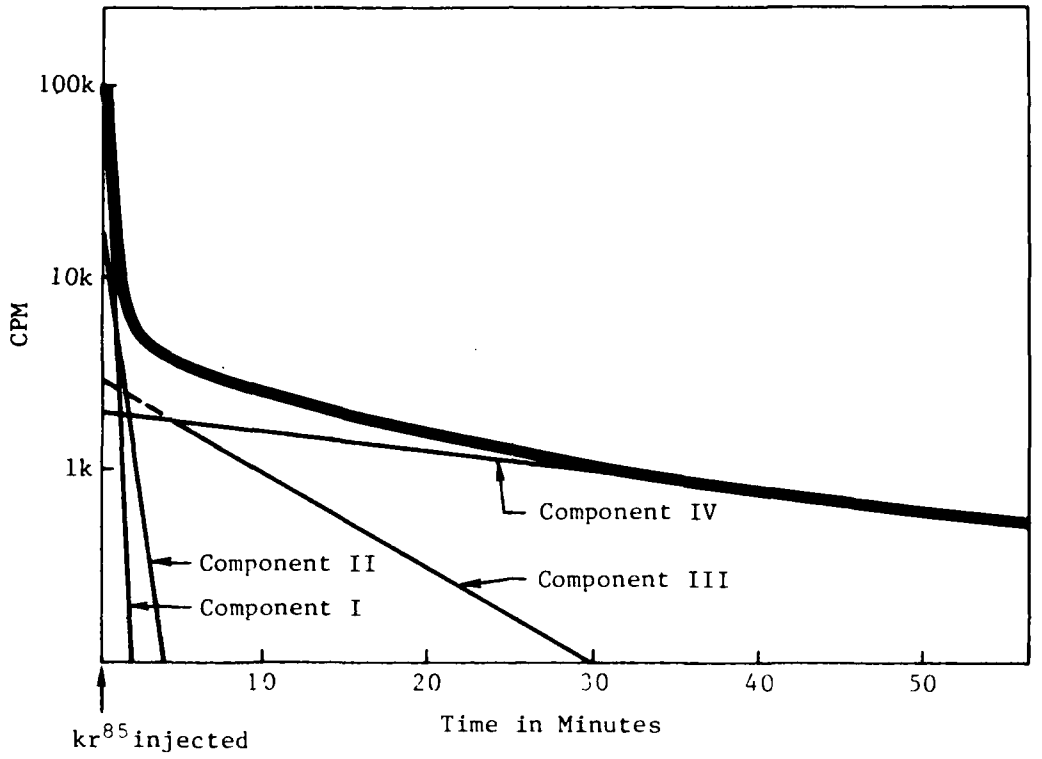
^{85}Kr is an ideal substance for such measurement. Not only is it inert, lipid-soluble and highly diffusible, but because of its low solubility in blood relative to air, 95% or more is removed in one circulation through the lung, so that the amount returning to the kidney is negligible (Thorburn et al., 1963).

A typical ^{85}Kr washout curve is presented in Figure 5-1. The disappearance of radioactivity is a complex function of time and can be described by a series of exponentials, each associated with blood flow through localized regions of the kidney. These regions have been identified by autoradiographic techniques such as: I. the outer cortex; II. the inner cortex and outer medulla; III. the inner medulla; and IV. the perirenal and hilar fat (Pitts, 1974; Thorburn et al., 1963). From the partition coefficient for ^{85}Kr, the slope of each exponential, and the density of the tissue, blood flow per gram of tissue can be calculated for the four differently perfused masses of renal tissue by the following equation:

$$F = \frac{\lambda k}{\rho}$$

where F = flow in ml/min/g; k = the slope of the exponential $\frac{0.693}{t_{1/2}}$ in min^{-1}; λ = the tissue/blood partition coefficient; and ρ = the specific gravity of the tissue in g./ml. (Thorburn et al., 1963). Total renal blood flow can be calculated by using the initial slope of the original washout curve (Ayer et al., 1971).

The partition coefficient λ is generally considered as 1 in the equations relating to the three compartments of the renal parenchyma (exponentials I - III); when dealing with the fourth compartment, a fat/blood partition coefficient of 9 is utilized (Thorburn et al., 1963).



Typical ^{85}Kr disappearance curve (heavy black curve) following injection of the isotope into the renal artery. Graphic analysis of the resultant exponentials is shown by thinner lines: I, outer cortex; II, inner cortex and outer medulla; III, inner medulla; IV, perirenal and hilar fat flow.

Modified from Pitts, 1974

FIGURE 5-1
^{85}Kr DISAPPEARANCE CURVE

The intercept of each exponential with the y axis represents the counts per minute initially present in compartment I - IV. The percentage of total initial counts represented by each of these values is equivalent to the fraction of total renal blood flow supplied to the respective compartments or the intrarenal distribution of blood flow.

Xenon 133 (^{133}Xe) has also been used for determining intrarenal hemodynamics; however, values for regional blood flow obtained by the washout of this radioactive gas are not comparable to those obtained using ^{85}Kr. One factor contributing to the observed differences may be the apparent variation of the ^{133}Xe tissue/blood partition coefficient with the hematocrit. Since the renal hematocrit varies from one region of the kidney to another, one cannot use a fixed value for λ in the calculation of flow rate (Carriere, 1970).* In addition, resolution of the washout curve into its component exponentials is noticeably more difficult for ^{133}Xe than for ^{85}Kr and therefore subject to greater error (Carriere, 1970). Since the tissue/blood partition coefficient for ^{85}Kr does not vary with the hematocrit, and since the analysis of the composite washout curve into its component exponentials is less subject to error for ^{85}Kr than for ^{133}Xe, the use of ^{85}Kr may be preferable (Carriere, 1970). Furthermore, if autoradiograms are desired in order to

*The tissue/blood partition coefficient for ^{133}Xe is usually taken as 0.65 (Carriere, 1970).

interpret an abnormal washout curve, ^{85}Kr must be used for the washout curve or injected subsequent to ^{133}Xe washout studies since ^{133}Xe cannot be utilized for autoradiographic analysis (Ayer et al., 1971; Carriere, 1970).

A nonradioactive washout technique has been used (Churchill et al., 1977) in which tissue hydrogen concentrations in the cortex are measured using surgically placed platinum electrodes. In these measurements, the experimental animal inhales hydrogen gas and the half-time of hydrogen desaturation in the cortical tissue is determined by graphic analysis of the monoexponential decay curve measured amperometrically from the electrodes. The cortical blood flow rates are then calculated as described earlier in this section. This technique has the advantage that the anatomical department monitored is determined by the placement of the electrodes and therefore does not require the analysis of complex exponential decay curves as ^{85}Kr washout techniques. The major disadvantage of the technique is that the electrodes require surgical placement, which may alter the local blood flow because of tissue injury (Churchill et al., 1977).

Washout techniques for determining intrarenal hemodynamics are most commonly performed in rats and dogs (Ayer et al., 1971; Churchill et al., 1977). Normal values in dogs for blood flow through the four regions defined by the ^{85}Kr washout curve are presented in Table 5-1, along with the percent distribution of blood flow in each

TABLE 5-1

REGIONAL BLOOD FLOW AND INTRARENAL DISTRIBUTION
OF BLOOD FLOW IN NORMAL DOGS

Exponential	Compartment	Regional Blood Flow[a] (ml/min/g)	Percent of Total Blood Flow[b]
I	outer cortex	4 - 5	80
II	inner cortex/ outer medulla	1.2 - 0.20	16
III	inner medulla	0.12 - 0.20	2
IV	perirenal and hilar fat	0.02	2

[a]Pitts, 1974
[b]Thorburn et al., 1963

region. These values are reproducible and are consistent with those determined by other means (Carriere, 1970; also see Section 5.2.2).

The ^{85}Kr washout technique appears promising as a level II screening test for both chronic and acute studies in dogs since catheters may be permanently implanted in the renal artery of anesthetized animals and, following a recovery period of approximately one week, blood-flow measurements may be performed repeatedly in the absence of anesthesia. Thorburn et al. (1963) reported performing repeated measurements in chronically catheterized dogs for as long as one year. Studies in rats can be performed only once since they involve abdominal incision and isolation of the kidney for counting (Ayer et al., 1971). Thus, rats are suitable for acute exposure studies in which a control value is obtained immediately prior to administration of the nephrotoxin and effects are determined in the same animals within a few hours. For chronic exposure studies, control values would have to be obtained using a separate group of animals.

Changes in intrarenal distribution of blood flow or reductions in regional blood flow as determined by washout techniques appear to be useful indicators of nephrotoxicity when the nephrotoxin alters renal hemodynamics. For example, the antitumor agent inosine dialdehyde exhibits dose-limiting renal toxicity. Kaufman et al. (1977) utilized a ^{133}Xe washout technique in conjunction with O ^{131}IH disappearance and renal biopsy to show that the renal impairment in

dogs was tubular necrosis due to a reduction in renal blood flow by the drug. While a dose of 20 mg./kg./day inosine dialdehyde for 7 days produced no significant changes in blood flow as determined by ^{133}Xe washout, a dose of 40 mg./kg./day for 5 days resulted in a reduction to 80% of the control value, and a dose of 60 mg./kg./day resulted in a further reduction to 57% of the control value. In both cases, renal biopsy findings confirmed structural alterations in the renal tubules.

In another study, Ayer et al. (1971) utilized ^{133}Xe washout and ^{85}Kr autoradiography to determine alterations in regional renal hemodynamics in rats resulting from glycerol-induced acute renal failure.

The parameters investigated were: total renal blood flow in ml./min./g. of total kidney; regional blood flow in ml./min./g. of compartment tissue; intrarenal distribution of blood flow, and true regional blood flow in ml./min./g. of total kidney.*

The results of the study are presented in Table 5-2 and serve to illustrate the type of information obtained from washout studies. The acute effects of the nephrotoxin were manifested as a steady decline in total RBF from 3.43 ml./min./g. to 0.92 ml./min./g. or 27% of the control value in 24 hours. A marked redistribution of blood flow also occurred, with the fraction supplied to the outer cortex

*True regional blood flow may be obtained by multiplying total renal blood flow by percent distribution of blood flow (Ayer et al., 1971).

TABLE 5-2

INTRARENAL HEMODYNAMICS IN RATS AS DETERMINED BY ^{133}Xe WASHOUT FOLLOWING GLYCEROL INDUCED ACUTE RENAL FAILURE

	Total RBF (ml/min/g kidney)	Regional Blood Flow (ml/min/g component)		Percent total RBF to component			True Regional Flow[a] (ml/min/g kidney)	
		I	II	I	II	III & IV	I	II
Control	3.43	4.55	1.12	78.8	15.7	5.5	2.70	0.54
10 minutes after glycerol	2.79	4.13	1.07	67.4	25.0	7.6	1.88	0.70
2 hours after glycerol	1.92	3.99	1.08	48.5	41.0	10.5	0.93	0.79
24 hours after glycerol	0.92	4.28	0.80	24.4	43.9	31.7	0.22	0.40

[a]True Regional Blood Flow = Total RBF x percent Total RBF to component

SOURCE: Ayer et al. 1971

decreasing from 78.8% to 24.4% after 24 hours, and the fractions supplied to the inner cortex/outer medulla and inner medulla/perirenal and hilar fat increasing from 15.7% to 43.9% and 5.5% to 31.7% respectively in that same period. True regional blood flow decreased dramatically in the outer cortex to 8% of the control value in 24 hours and, despite the increase in the fraction of blood flow to component II, true regional blood flow in that component decreased to 74% of the control value.

^{85}Kr autoradiographs of control and experimental animals 24 hours following glycerol administration are presented in Figure 5-2 (Ayer et al., 1971).

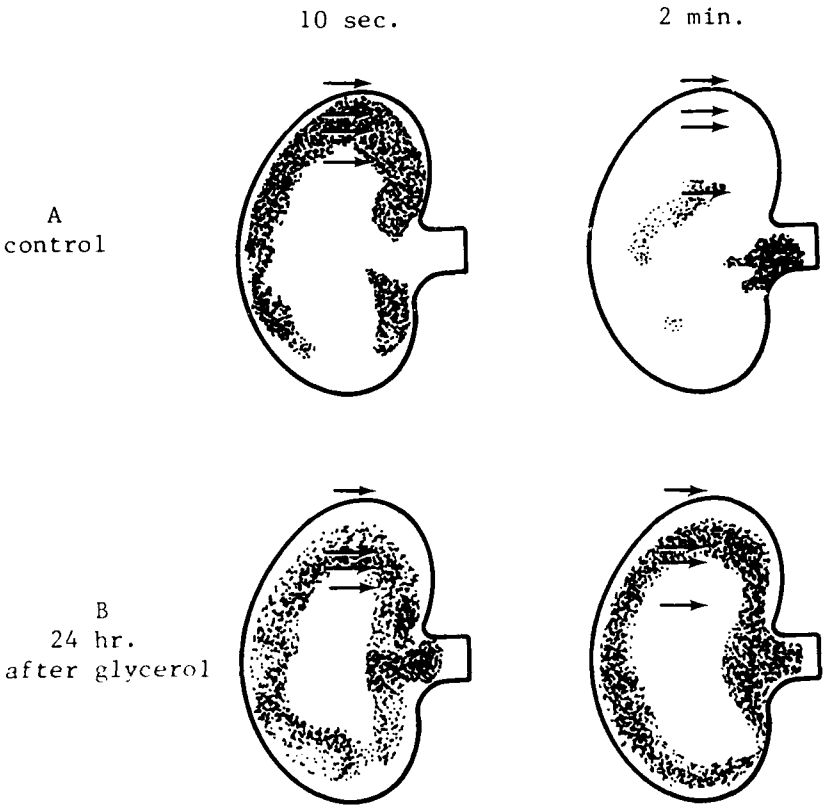
Critics of washout techniques point out several theoretical considerations which may affect the accuracy and interpretation of the results (Katz et al., 1971; Stein et al., 1973). These include:

- no direct evidence for an equilibrium distribution of the indicator gas between tissue and capillary blood
- the assumption that λ and V, the volume of distribution of the gas, are constant, and
- the subjective nature of the analysis of the washout curve into its component exponentials, although this is not a problem in H_2 washout.

However, as was stated before, the values obtained by ^{85}Kr washout appear to agree well with those obtained by other means, and the procedures are nondestructive in dogs and may be repeated frequently.

5.2.2 Microsphere Techniques

The extraction and entrapment of radioactive microspheres by the glomerular capillaries may be utilized for the determination of

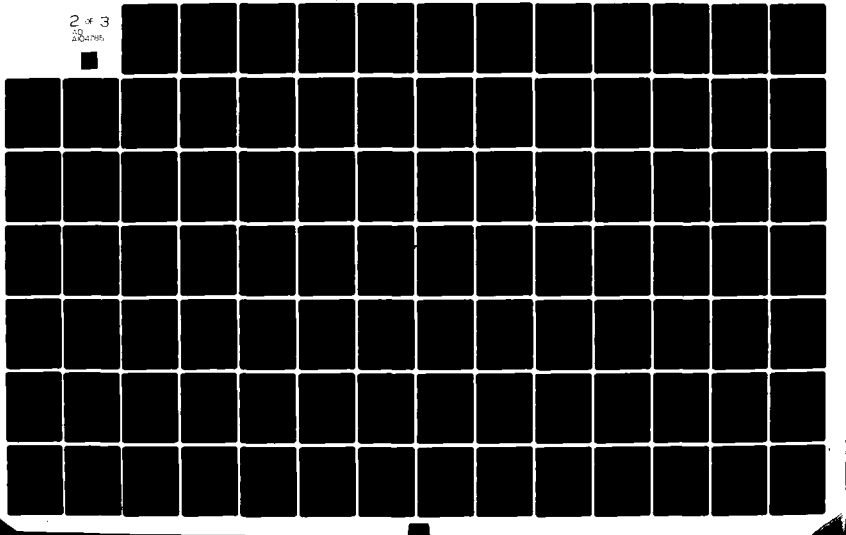


^{85}Krypton autoradiographs taken 10 seconds and 2 minutes after injection of the indicator gas. Arrows indicate (from the surface toward the papilla) the position of the renal capsule and the boundaries between cortex and subcortical area, subcortical area and outer medulla, outer medulla and inner medulla. An outline of the kidney is provided indicating the approximate surface area to aid in studying the autoradiographs. Note the delayed, weak, and patchy filling of the cortex and the delayed washout from the cortex 24 hours after glycerol injection.

FIGURE 5-2
^{85}KRYPTON AUTORADIOGRAPHS

Modified from Ager et al., 1971

AD-A104 765 MITRE CORP MCLEAN VA METREK DIV F/G 6/20
EVALUATION OF SHORT-TERM BIOASSAYS TO PREDICT FUNCTIONAL IMPAIR--ETC(U)
OCT 80 B FULLER, R THOMAS, J KONZ, L THOMAS DAMD17-78-C-8068
UNCLASSIFIED MTR-80W00234 NL



regional blood flow and intrarenal distribution of blood flow. However, since the glomeruli are concentrated within the cortex of the kidney, these parameters can only be measured within the various sections of cortical tissue using this technique.

In this procedure, a bolus of microspheres is injected into the left ventricle or root of the aorta of an anesthetized animal. Complete mixing occurs before the microspheres reach the renal artery, so that the quantity of microspheres entering the renal tissue is a function of the fraction of blood flow to the tissue. If all the microspheres are trapped in the glomerular capillaries in a single circulation through the kidney, the blood flow, F, in ml/min/g of tissue can be calculated from the following formula:

$$F = \frac{q}{Q\,w} \times RBF$$

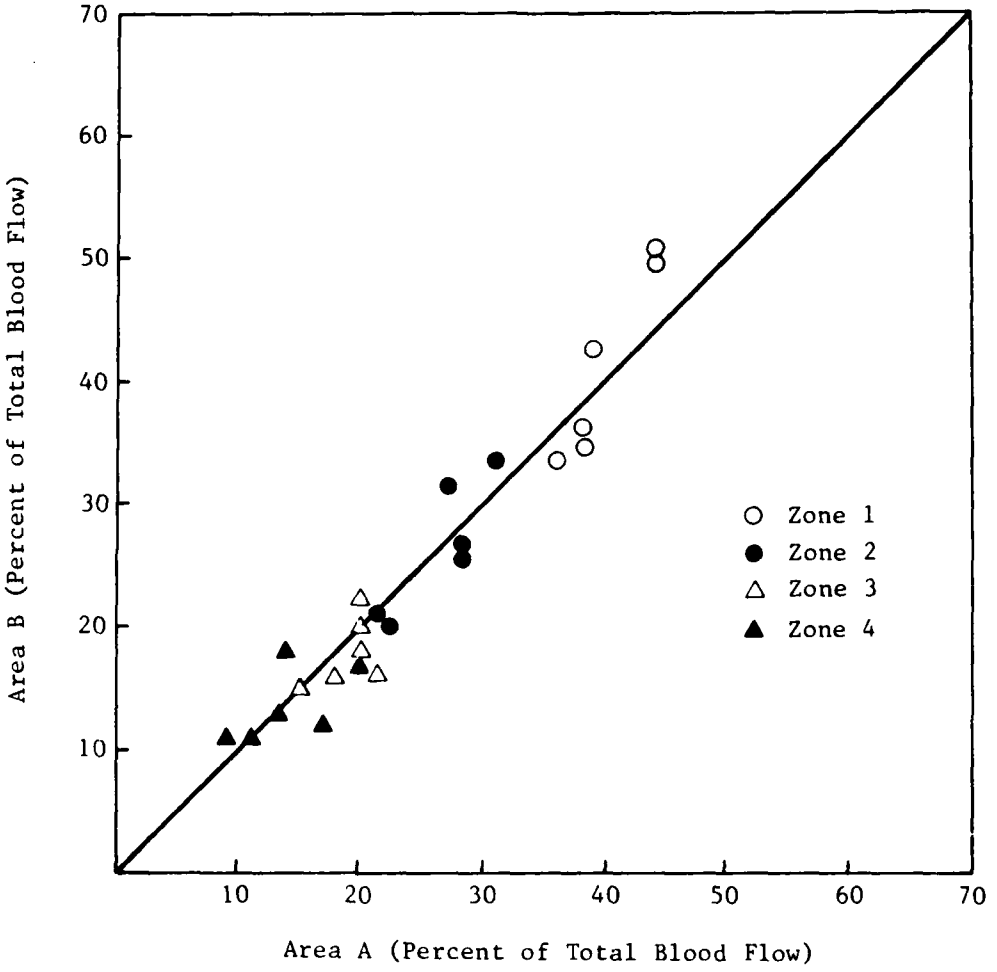
where q = the counts per minute in a tissue slice of weight w, Q = the counts per minute in the entire kidney and RBF = the renal blood flow as measured by standard techniques (see Section 5.1 [Blantz et al., 1971; Katz et al., 1971 Slotkoff et al., 1971]). Plastic microspheres of approximately 15μ in diameter and labelled with ^{169}Yb, ^{85}Sr, ^{46}Sc, or ^{141}Ce are most commonly used for blood flow determinations. Microspheres of this size are suitable in that they:

- are the smallest beads completely extracted by the glomerular capillaries in one circulation
- do not damage renal tissue
- do not alter blood flow to the kidney

- do not alter any physiological functions, and
- have rheologic properties similar to those of blood (Katz et al., 1971).

Microspheres have been utilized for determining cortical hemodynamics in both rats and dogs. Following injection of the labelled spheres, the experimental animal is sacrificed and the kidneys are removed and weighed. One half of one kidney is weighted again, cut into small pieces and counted in a scintillation counter. From the kidney weights and the CPM in one half kidney, the total CPM per kidney, Q, is determined. The other half kidney is used to prepare slices of cortex. These slices are cut with a microtome in successive layers from the superficial to the deeper areas of the cortical tissue. Each slice is then weighed and counted to obtain values for q and w (Katz et al., 1971).

Stein et al. (1973) developed a procedure for cutting the renal cortex into four equidistant sections which were representative of superficial nephrons (Zone 1), midcortical nephrons (Zones 2 and 3) and juxtamedullary nephrons (Zone 4). The fractional distribution of blood flow between the four zones in two different areas of renal cortex are presented in Figure 5-3. As can be seen from the figure, definite differences in flow are present in the various cortical zones, with over two-thirds of the blood flow occurring in the two outer zones. Stein et al. (1973) indicate that blood flow in zones 1 through 3, as determined by the microsphere technique, is equivalent to outer cortical blood flow (C_I), as determined by the washout



Stein et al., 1973

FIGURE 5-3
COMPARISON OF THE FRACTIONAL DISTRIBUTION OF BLOOD FLOW BETWEEN TWO DIFFERENT AREAS OF THE RENAL CORTEX AFTER A SINGLE INJECTION OF RADIOACTIVE MICROSPHERES

technique. Outer cortical blood flow in dogs, as determined by the microsphere technique, is approximately 4.5 ml./min./g. and agrees well with the range of 4-5 ml./min./g., as determined by washout techniques (Slotkoff et al., 1971; Pitts, 1974).

Since entrapment of microspheres in the glomerular capillaries does not interfere with blood flow or alter physiological functions, this method of measuring changes in cortical hemodynamics can be useful for screening nephrotoxins. Control values can be determined by using microspheres labelled with one of the four previously mentioned radioisotopes. Changes in blood flow following administration of the nephrotoxin can be measured subsequently using microspheres labelled with a different isotope. Slotkoff et al. (1971) determined that C_{IN} and C_{PAH} remained unchanged after four consecutive injections of microspheres; however, the maximum period of time over which consecutive measurements may be performed is yet to be determined. The tests are therefore definitely applicable to acute toxicity screening and possibly applicable to chronic studies as well.

While microsphere techniques have not been utilized for nephrotoxicity screening to date, their ability to detect changes in blood flow under a variety of experimental conditions (e.g., acute hemorrhage) has been demonstrated (Blantz et al., 1971; Katz et al., 1971; Slotkoff et al., 1971; Stein et al., 1973). Stein et al. (1973), for example, found that the renal vasodilator acetylcholine causes a redistribution of cortical blood flow from outer to inner cortical

nephrons. The fractional distribution of flow in Zone 1 fell from 45% to 33% and was accompanied by increased flows in Zones 3 (17% to 23%) and 4 (10% to 16%). Absolute flow in all 4 zones was also found to increase. The same laboratory using microsphere techniques found that hemorrhagic hypotension caused a marked redistribution of cortical flow to the inner cortical layers, while the vasopressor norepinephrine reduced total blood flow to the same extent, but had no effect on the zonal distribution of blood flow. Redistribution of cortical circulation during acute hemorrhage was also reported by Slotkoff et al. (1971) in a study designed to confirm the utility of the microsphere technique. In this study the ratio of outer cortical to inner cortical radioactivity decreased from 3.00 before hemorrhage to 1.3 after hemorrhage. It therefore appears likely that microsphere techniques for measuring regional blood flow and intrarenal distribution of blood flow, like washout methods, might be useful in assessing nephrotoxicity in those instances where the nephrotoxin alters regional hemodynamics.

5.3 Summary

Several procedures are available for measuring renal blood flow (RBF) and intrarenal distribution of blood flow in laboratory animals, including rats and dogs. These procedures are not suitable for Level I of a tiered screening program for nephrotoxicity; however, the more developed tests might be appropriately included in Levels II

or III of such a program and are useful in detecting renal damage from nephrotoxins which alter renal hemodynamics.

The effective renal plasma flow (ERPF) may be determined by standard clearance procedures employing radiolabelled p-aminohippuric acid (PAH), orthoiodohippurate (OIH) and iodopyracet (Diodrast); or by plasma disappearance methods employing radiolabelled OIH or Diodrast.

Clearance procedures for measuring ERPF, like clearance procedures for measuring glomerular filtration rate (GFR), require collection of timed urine samples (as well as timed blood samples) and thus bladder catheterization. Intravenous infusion is necessary for all three marker substances.

Plasma disappearance methods for determining ERPF are less cumbersome than plasma clearance methods, as they involve only a single injection of the marker followed by collection of timed blood samples.

Since nephrotoxins may markedly alter the extraction ratio, E, accurate determination of RBF by either plasma clearance or plasma disappearance methods requires simultaneous determination of E which, in turn, requires renal vein catheterization. Determination of RBF in laboratory animals is therefore somewhat more complicated than determination of GFR by a similar procedure.

Regional blood flow and intrarenal distribution of blood flow may be determined by measuring the washout of Krypton-85 (^{85}Kr),

Zenon-133 (^{133}Xe) or hydrogen from the kidney, or by measuring the extraction and entrapment of radioactive microspheres by the glomerular capillaries. Washout techniques involve either the rapid injection of the indicator gas into the renal artery, followed by monitoring the loss of radioactivity over the kidney by an external scintillation detector, or the inhalation of a gas (hydrogen) and the electrode monitoring of cortical tissue desaturation. For the radioactive gas washout, the resulting curve can be described by a series of exponentials, each associated with blood flow through localized regions of the kidneys. Hydrogen washout is described by a monoexponential decay curve. In the radioactive gas washout curve, the intercept of each exponential with the y axis represents the CPM initially present in the corresponding region and can be related to the fraction of total blood flow in that region. In hydrogen washout technique, the placement of the electrodes determines the anatomical department monitored.

Washout techniques have been utilized in both dogs and rats. Dogs may be chronically catheterized, permitting repeated measurements to be performed over extended periods of time, and are thus suitable for chronic as well as acute studies. Studies in rats involve exposing the kidney for counting, and can be performed only once. Rats are therefore suitable for acute studies or for chronic studies if control values are obtained in a separate group of animals.

Cortical blood flow and distribution of blood flow within the cortex can be measured in rats and dogs using the microsphere technique. In this procedure, a bolus of radioactive microspheres is injected into the left ventricle or the root of the aorta, and the distribution of radioactivity within the various layers of cortical tissue is determined by direct count. Although the microsphere technique requires sacrifice of the animal, comparison of cortical hemodynamics before and after exposure to a nephrotoxin is possible through sequential measurements utilizing microspheres labelled with different isotopes.

6.0 ENZYMATIC INDICATORS OF DAMAGE

Enzyme determinations on serum have become an important and widely accepted tool for the diagnosis of many diseases. Measurement of urinary enzymes has not received the same amount of attention until very recently, and the diagnostic value of urinary enzymatic determinations is still controversial. However, as an indicator of renal functional impairment, urinary enzyme determinations have several advantages over the use of serum enzyme determinations.

Increased enzyme activity can be measured in the serum, but it is not a sensitive index of renal damage since other body systems can also make an important contribution to the serum level of some enzymes. Experimental evidence has shown that when tubular cells degenerate, the enzymes contained in the cells pass into the urine (Raab, 1969c); following administration of nephrotoxins, urinary activity of enzymes derived from kidney cells was found to be highly increased (Raab et al., 1969). Since most urinary enzymes are derived from renal tubular cells (Rabb, 1968), changes in the urinary excretion rates more closely reflect alterations in tubular cells. Therefore, elevated urinary enzyme activity is regarded as a sensitive indicator of tubular injury (Mattenheimer, 1968; Ellis et al., 1973c).

6.1 Urinary Enzyme Activity

Each of the distinct regions of the nephron has a characteristic group of enzymes. It is believed that changes in the output of

urinary enzymes reflect changes in the anatomical nature of the nephron. Because of the large functional reserve capacity of the kidney, variations in urinary enzyme activity are believed to occur before changes in physiological function (Schoenfeld, 1965). Therefore, measurement of urinary enzyme activity should provide a sensitive early indicator of renal damage. In addition, it is possible to indicate which portion of the nephron is damaged since different parts of the nephron show widely differing levels of enzyme activity (Ellis et al., 1973c; Davison and Conning, 1968). Thus assays of specific enzymes are capable of differentiating different parts of the nephron; changes in the activity of those specific enzymes can be indicative of effects to specific sections of the nephron.

Most chemically-induced lesions have been found in the proximal convoluted tubules (Balazs et al., 1963). Renal tubular cells contain high levels of many enzymes in order to fulfill their numerous biochemical functions (Raab, 1968; Ellis et al., 1973a, 1973b). Because the tubular cells are rich in enzymes, urinary enzyme activity is markedly increased when these cells disintegrate. The excretion pattern of renal enzymes following toxic damage to tubular cells depends on such factors as (1) intensity of damage, (2) enzyme distribution in the damaged parts of the nephron, (3) localization of the enzymes within the damaged cells, (4) structures to which the enzymes are bound, and (5) the physical properties of the enzyme molecules (Raab, 1972).

More than forty different enzyme activities have been detected in the urine of mammals (Raab, 1972). These include representatives of four groups--oxydoreductases, transferases, hydrolases and lyases. A list of these enzymes has been compiled in Table 6-1. Appendix E provides an additional listing along with specific references.

Although many enzymes have been detected in the urine of mammals, relatively few have been studied to any extent. The following sections will be limited to a discussion of those urinary enzymes that are most frequently measured to indicate renal damage. These include, in decreasing order of usefulness in detecting damage: beta-glycosidases, alkaline phosphatase, lactic acid dehydrogenase (LDH), acid phosphatase, glatamic oxaloacetic transaminase (GOT), cholinesterase and isocitrate dehydrogenase (ICDH).

All the urinary enzyme assays discussed in the following sections are standard tests. Although these tests are not routinely employed for initial screening in toxicity testing, they can be used as screening tests or to further define the location of the toxic reaction. These tests are relatively simple, easy to perform, reproducible and less expensive than most of the methods used to assess renal dysfunction. The major drawback is collecting serial, uncontaminated urine samples from the small test animal. Preserving the specimens and dialyzing the samples before the tests are performed are also necessary.

TABLE 6-1

URINARY ENZYMES FOUND IN HUMANS AND IN ANIMALS

ACTIVITY	HUMAN	ANIMAL
1. Oxidoreductases		
Lactate dehydrogenase,	+	+
Malate dehydrogenase,	+b	+b
Isocitrate dehydrogenase,	-	+
Glucose-6-phosphate dehydrogenase,		+
Succinate dehydrogenase,	-	-
Glutamate dehydrogenase,	-	+
Diamino-oxidase,		+
Dihydroxphenylalanine oxidase	+	
Catalase,	+b	
2. Transferases		
D-glutamyltransferases,	+	
Aspartate aminotransferase (GOT),	+	+
Alanine aminotransferase (GPT),	+b	+
Ribonuclease,	+	+
Arginine-ornithine-transamidinase,	+b	
3. Hydrolases		
Triglyceride esterase (lipase),	+	+
L-gulono-δ-lactone hydrolase,		+
Cholinesterase,	+	+
"Alkaline" phosphatase,	+	+
"Acid" phosphatase,	+	+
"Acid" deoxyribonuclease,	+	+
"Neutral" deoxyribonuclease,	+	+
Sulfatases,	+	+
Amylase,	+	+
Muramidase,	+	+
α-Glucosidase,	+	+
β-Glucosidase,	+	+
β-Galactosidase,	+	+
Trehalase,	+	
N-Acetyl-d-glucosamidase,	+b	+b
β-Glucurondase	+	+

TABLE 6-1 (Concluded)

URINARY ENZYMES FOUND IN HUMANS AND IN ANIMALS

ACTIVITY	HUMAN	ANIMAL
3. Hydrolases (Conc.)		
Aminopeptidases, ("Leucine aminopeptidase;" glycine aminopeptidase; cystine aminopeptidase, Alanine aminopeptidase)	+	+
Carboxypeptidase, (Carboxypeptidase B)	+	+
Renin,	+	+
Proteases: (tryptic activity (fibrinolytic activity); catheptic activity; peptic activity; kallidrein, urokinase)	+	+
4. Lyases		
Aldolase,	+b	
Hyaluronidase	+	+

[a](+ = found, - = not found, blank = no information available).
[b]Under pathological conditions only.

SOURCE: Raab, 1972.

Assays of urinary enzyme activity require only a dialyzed urine sample and a standard spectrophotometer and/or colorimeter of the type routinely available in toxicology laboratories. These assays do not require extensive training to perform.

6.1.1 Beta-Glycosidases

The assay of beta-glycosidases is a useful tool for detecting early renal tubular damage in rats (Coonrod and Paterson, 1969a; Patel et al. 1975; Price and Dance 1967; Robinson et al., 1967a) and dogs (Ellis et al., 1973a, 1973b, 1973c).

Renal tubular damage induced by administration of uranium nitrate, mercuric chloride, potassium dichromate or 4-nitrophenyl-arsonic acid caused a marked increase in the urinary excretion of beta-glucosidase, beta-glucuronidase, beta-galactosidase and N-acetyl-beta-glucosaminidase in rats due to breakdown of the renal tubular cells (Price et al., 1971; Robinson et al., 1967a). Several other nephrotoxic agents (e.g., gentamicin, ethyleneimine) also induce an increased excretion of beta-glycosidases in the urine of rats and dogs (Ellis et al., 1973b; Patel et al., 1975).

Coonrod and Paterson (1969a, 1969b) found consistent increases in urinary beta-glucuronidase activity in rats after extirpation of the preputial glands when the rats were treated with mercuric chloride, causing tubular injury. These authors suggested that beta-glucuronidase should not be used to assess renal tubular damage

unless the preputial glands have been extirpated, because there is a marked release of this enzyme from these glands.

The assay of beta-glucuronidase activity in the urine of rats free of bladder tumors is a simple, inexpensive and reproducible indicator of renal tubular damage (Coonrod and Paterson, 1969a, 1969b).

6.1.2 Alkaline Phosphatase

Using biochemical and histochemical procedures, it has been shown that a high concentration of alkaline phosphatase is present in the renal tubules of rats, rabbits and dogs (Gomori, 1939, 1946; Lunseth, 1960). Several studies performed in animals and humans (Amador et al., 1965; Asscher and Anson, 1960; Breedis et al., 1943; Lunseth, 1960; Raab, 1969d; Raab et al., 1969) indicated that, following the administration of nephrotoxic agents, renal ischemia or radiation toxicity, alkaline phosphatase decreased in the renal tissue and increased considerably in the urine. These increases in alkaline phosphatase activity in the urine are thought to be indicative of proximal tubular injury (Breedis et al., 1943; Nomiyama et al., 1973). In addition, increased urinary activity of alkaline phosphatase is known to result from a wide variety of urinary tract diseases (Wilkinson, 1968).

The urinary alkaline phosphatase assay procedure is highly precise and repeatable (Amador et al., 1963a, 1963b), and even small elevations of this enzyme in the urine can be easily detected. However, urine samples must be dialyzed to remove enzyme inhibitors.

6.1.3 Lactic Acid Dehydrogenase (LDH)

Determinations of urinary LDH activity can be used for screening nephrotoxic action of substances in rats, rabbits, dogs and cats (Raab, 1968; Raab et al., 1969). Such renal diseases as active glomerulonephritis, acute tubular necrosis, acute renal infarction, necrotizing vascular disease and pyelonephritis (Wacker and Dorfman, 1962) have been shown to produce elevated urinary LDH activity.

Besides the fluorimetric method, several other procedures have been suggested by Raab (1968) for determining LDH activity in the urine. Best results are obtained with the direct photometric determination of the conversion of NAD to $NADH_2$ in the presence of pyruvate. Another method is based on the reduction of a red indophenol dye by the hydrogen acceptor. The colorimetric determination of LDH activity with dinitrophenylhydrazine can be used, but it is not very suitable for this purpose (Raab, 1968). In all animal species, LDH inhibitors are present in the urine (Wacker and Dorfman, 1962). Dialysis of urine against water or gel filtration should be performed for the removal of such inhibitors. Some chemotherapeutic agents that are excreted in the urine, such as nitrofurantoin, interfere with the determination of LDH activity and must be taken into consideration.

Increased excretion of LDH in the urine can indicate renal tubular damage (Raab, 1968). However, disease states in other organs

can produce elevated levels as well, thus limiting the applicability of this test (Gault and Geggie, 1969; Raab, 1968).

6.1.4 Acid Phosphatase

Urinary acid phosphatase activity has been measured in rats following exposure to methyl mercury and mercuric chloride (Stroo and Hook, 1977b) and in dogs treated with mercuric chloride and ethylenimine (Ellis et al., 1973a, 1973b, 1973c). The determination of acid phosphatase in the urine is a test of anatomical integrity according to Schoenfeld (1965). This is because the renal tubules are rich in acid phosphatase, and this enzyme is distributed widely throughout the nephrons. Urinary acid phosphatase may also be related to enzymic activity in the serum because the low molecular weight of the enzyme permits glomerular filtration (Raab, 1968); however, because its urine concentration is substantial, contamination with small amounts of blood has little influence on urinary activity (Schoenfeld, 1965). In addition, prostatic secretion contributes to a marked extent to acid phosphatase activity in the urine of male animals. This is the only enzyme that shows higher activity in glomeruli than in tubular cells. Therefore, increased urinary acid phosphatase activity does not give a true picture of renal tubular damage. However, since it is present throughout the nephron, any nephropathy can cause increased urinary activity of the enzyme. Even though urinary acid phosphatase lacks sensitivity, it is considered a suitable measure for general screening purposes (Schoenfeld, 1965).

6.1.5 Glutamic Oxaloacetic Transaminase (GOT)

Measurement of urinary GOT is a reliable procedure for detecting tubular injury and is also a useful tool for comparing studies of tubular nephrotoxicities in animals such as rats, rabbits and cats (Balazs et al., 1963; Kemp and Laursen, 1960). In these studies, nephrotoxicity was induced by total ischemia, sodium chromate, uranyl nitrate and mercuric chloride. GOT is either not excreted at all or excreted in very small amounts in the urine of normal animals; but after administration of nephrotoxicants or induction of renal ischemia, it is excreted in increased amounts in the urine. The increased urinary GOT levels indicate renal cellular injury in animals; higher plasma levels of this enzyme do not lead to higher urinary levels when the glomerular membrane is intact (Kemp and Laursen, 1960).

It is common to measure GOT activity in blood samples; however, the assay of urinary GOT activity is not considered a routine measure. The test may be used for screening purposes, but other urinary enzyme determinations are more commonly used as an indicator of tubular damage.

6.1.6 Cholinesterase and Isocitrate Dehydrogenase (ICDH)

Both cholinesterase and ICDH are present at very low levels in the urine of rats. Raab (1969a) found a high cholinesterase activity in the urine of rats when tubular cells containing high activities of cholinesterase were destroyed in nephrotic damage. Increased urinary

ICDH activity in rats was also reported by Raab (1969c). Vascular shock, produced by an anaphylactoid agent (Compound 48/80),* elevated ICDH activity due to hypoxic damage of the renal system. Similarly, in toxic nephrosis caused by sodium tetrathionate or d-serine, ICDH levels were increased in the urine of rats because the enzyme present in degenerating tubular cells is excreted through the urine. These tests appear to be sensitive indicators of tubular damage. They are not tests which are routinely performed to assay kidney abnormalities, but can be used when dysfunctions of other organs are ruled out.

6.1.7 Multiple Enzyme Determinations

In rabbit studies, Nomiyama et al. (1974) observed that the determination of several urinary enzymes, alkaline phosphatase, GOT and glutamic pyruvate transaminase (GPT), was more useful in detecting early renal injury than renal function tests. The most probable cause of the observed enzymuria was release of enzymes from destroyed tubular cells. Proximal tubular injury was believed to be indicated by increased alkaline phosphatase activity, while distal tubular injury resulted in the elevation of urinary LDH activity.

Increased urinary activity of maltase and alkaline phosphatase was reported in rats following treatment with a known nephrotoxin, mercuric chloride. It was suggested that this increased activity

*The chemical identity of Compound 48/80 was not reported in the literature.

could be used as an index of renal damage; however, the authors point out that this measure may be no more sensitive than currently utilized methods (e.g., specific-gravity or osmolality, PAH transport), (Stroo and Hook, 1977a).

Simultaneous measurements of LDH and alkaline phosphatase activities can be used for differential diagnosis of renal diseases and in tracing their evolution, according to Amador et al. (1965). In their studies, most patients studied who had potentially fatal renal diseases had elevated urinary LDH activity, while only certain types of renal disease produced elevated urinary alkaline phosphatase activity. This combination of measurements should provide similar information on the site of injury in experimental animals used in short-term chemical toxicity testing.

Nomiyama et al. (1973) point out the usefulness of using a battery of urinary enzyme determinations. In rabbit studies, they suggested that proximal tubular injury might be detected by a significant increase in urinary activity of alkaline phosphatase, accompanied by an increase in GOT, GPT and LDH, since these tubules are rich in these enzymes.

6.1.8 Other Enzymes

Several other enyzmes have been mentioned in the literature as possible indicators of renal damage. Since they are not frequently measured, only a brief discussion of two of them will be included.

Using histochemical staining techniques, leucine aminopeptidase (LAP) was found exclusively in the cortical tubules of rat kidney (Burstone and Falk, 1956), and several investigators suggest that the measurement of LAP in urine may be a sensitive indicator of renal tubular injury (Burstone and Falk, 1956; Mattenheimer, 1968; Raab, 1968; Raab et al., 1969; Diezi and Biollaz, 1979).

Several authors have discussed the use of urinary lysozyme determination as an indicator of renal damage (Diezi and Biollaz, 1979; Evan and Dail, 1974; Gault and Geggie, 1969; Patel et al., 1975). All agree that an increase in urinary lysozyme activity is associated with damage to the convoluted proximal tubules; however, Gault and Geggie (1969) and Patel et al. (1975) consider the assay to be of value for early detection of impaired tubular function, while Evan and Dail (1974) conclude that this test is indicative of severe cellular damage and cannot be considered an early indicator.

6.2 Summary

Damage to renal tubules may cause liberation of enzymes into the blood and urine. Determination of increased serum levels of these enzymes cannot be utilized as a sensitive indicator of renal tubular damage because other body organs can also make significant contributions to the serum levels of enzymes. However, measurement of urinary enzymes may be used as a sensitive index of renal tubular damage.

It should be pointed out that not all enzymes that are released from the breakdown of tubular cells appear in urine. This is due to such biochemical factors as stability at urinary pH and susceptibility to enzyme inhibitors (Raab, 1972). For example, even though histochemical methods showed a pronounced decrease in succinate dehydrogenase activity within the kidney cells following toxic kidney damage, no urinary activity for this enzyme could be detected (Raab et al., 1969). Furthermore, it should not be concluded that every elevation of urinary enzymatic activity proves the presence of nephrotoxic effect, since a substance that is secreted by the tubules may lead to increased activity by facilitating the permeation of enzymes into the tubular fluids (Raab, 1972). In addition, many factors, such as diseases in other organs and the presence of substances which influence diuresis, influence urinary enzyme activity (Raab, 1972).

In conclusion, the assay of urinary enzyme activities is a more sensitive technique for detecting some forms of early renal tubular damage than the commonly used functional tests (Diezi and Biollaz, 1979; Ellis et al., 1973a, 1973b) or histological examinations (Raab, 1972). However, there are sufficient difficulties in using urinary enzymes in routine screening that their application in the "routine evaluation of renal toxicity is still uncertain (Diezi and Biollaz, 1979)."

A few enzymes may have application in a screening program. The determination of urinary beta-glucosidase may provide a simple, sensitive means of detecting early tubular damage in rats and dogs when proper procedures and animal preparation are used. Measurements of urinary alkaline phosphatase, lactic acid dehydrogenase and acid phosphatase are less specific and give an indication of the general state of the kidney. Assays which appear to be useful for detecting early tubular damage include measurement of the urinary activities of glutamic oxaloacetic transaminase, cholinesterase and isocitrate dehydrogenase; however, these tests are not frequently used. The urinary enzymatic assays are relatively easy to perform and provide a noninvasive, nondestructive means of evaluating nephrotoxicity (Ellis et al., 1973a, 1973b; Nomiyama et al., 1973; Patel et al., 1975; Stroo and Hook, 1977a). These urinary enzyme determinations cannot be used to draw conclusions regarding the kind of damage present; nonetheless, the site of the damage may be indicated.

7.0 CONCLUSIONS AND RECOMMENDATIONS

The testing techniques for the assessment of renal toxicity have been classified in five categories: morphologic, glomerular filtration, tubular transport, renal hemodynamics and enzymatic indicators. The tests included in each of the five categories are shown in Table 7-1 and were categorized based on either structural, functional or biochemical alterations that can be determined using the different techniques.

The renal system performs many different functions related to glomerular filtration, tubular transport and blood flow. In so doing, it operates as three separate, but closely interacting, components: the glomerular component filters fluid and selective solutes, the tubular component reabsorbs and secretes nutrients and other substances, and the vascular component delivers blood to the nephron. A nephrotoxic substance may damage the glomeruli, tubules, effect blood flow or any combination of these. The various renal functions are localized in specific components of the renal system. The renal tests are generally applicable to only one type of renal function; however, there can be interactions between the three components of a damaged renal system. For these reasons, a number of tests are necessary in a screening program so that information can be obtained concerning all three components and their possible interactions.

TABLE 7-1

TESTS USED TO EVALUATE RENAL DAMAGE

TEST CATEGORY	SPECIFIC TESTS
Morphologic	• Gross Inspection • Light Microscopy • Electron Microscopy
Glomerular Filtration	• Blood Urea Nitrogen • Serum Creatinine • Urinary Protein • Inulin Clearance • Creatinine Clearance • ^{125}I-Iothalamate and ^{131}I-Diatrizoate Clearance • Plasma Disappearance of ^{125}I-Iothalamate and ^{131}I-Diatrizoate
Tubular Function	• Urinary Glucose • Glucose Transport Maximum • Urinary Acidification • PAH Transport Maximum • Renal Cortical Slices • Isolated Perfused Tubule Segments • Urinary Concentrating Ability - Specific Gravity - Osmolality • Urinary Diluting Ability • Microscopy of Urinary Sediment
Renal Hemodynamics	• PAH Clearance • Iodohippurate Clearance • Iodopyracet Clearance • Plasma Disappearance of either ^{125}I or ^{131}I labeled Iodohippurate and Iodopyracet • Kryton-85 Washout • Xenon-133 Washout • Hydrogen Washout • Krypton-85 Autoradiography • Radioactive Microsphere Distribution
Biochemical Indicators	• Urinary Enzyme Activity - beta-Glycosidase - beta-Glucuronidase - Alkaline Phosphatase (AlP) - Lactic Acid Dehydrogenase (LDH) - Acid Phosphatase (AP) - Glutamic Oxaloacetic Transminase (GOT) - Cholinesterase (CHE) - Isocitrate Dehydrogenase (ICDH)

On the basis of available information for each renal test, a tiered screening program is recommended for detecting renal damage in small laboratory animals. Evaluation of individual tests within each category is based on certain considerations. These considerations primarily include: validity of the measurement (e.g., sensitivity, accuracy, reproducibility); risk to life of the animal; costs of measurement (e.g., the costs of the necessary instrumentation, animals and labor); the time required to perform the test; and finally, significance with regard to reflecting renal damage. The selection criteria utilized to evaluate the renal tests are described in the following section.

7.1 Criteria Used in Evaluating Renal System Tests

The following criteria have been selected to evaluate each renal system testing technique for inclusion in a short-term screening program:

- State of development sufficient to be reproducible in a screening program
- Sensitivity sufficient to detect early subtle forms of damage or to provide an indication of the extent of damage to the system
- Procedures and instrumentation sufficiently uninvolved to enable technicians with some additional training to perform the tests, and
- Methods sufficiently brief so that each test can be completed within a few hours to a few days

Considerations that have also been used to evaluate the tests include (1) the availability of the animals used and whether the test

is terminal and (2) the costs of the test procedures; and animal, equipment and maintenance costs.

The species of animals used for screening affects both the cost and the validity of a particular measurement. The type and the number of animals used to perform an experiment affects the cost not only in terms of the actual cost of the animals, but also in terms of the time and labor required to perform the test. Also, the sensitivity, accuracy and reproducibility of a test will depend on the species in which the test is performed. However, there are not sufficient data available to establish very many of these relationships with regard to renal tests. Rats are the most common small laboratory animal used to evaluate the morphological and functional integrity of the renal system.

Sometimes anesthetized animals are used to perform a renal test. This has the benefit of reducing the time and difficulty in handling the animals, but anesthetic agents can affect renal functions. Whether or not the renal test is terminal is an important consideration if the intention is to perform serial or multiple determinations in an animal during a single experiment, or if the animal is to be utilized for more than one experiment.

Once the renal tests were evaluated for suitability in a short-term screening program and the selection made, the tests selected for use in the program were further subdivided into either of two levels of the tiered program, based upon the criteria shown in Table 7-2.

TABLE 7-2

CRITERIA FOR SHORT-TERM
RENAL TESTING TIERS

Criteria	Level I	Level II
State of Development	High	Moderate
Sensitivity	Moderate	Moderate to High
Indicates Extent of Damage	Moderat	Moderate to High
Complexity of Procedures and Instrumentation	Low	Moderate to High
Level of Skill	Low	Moderate to High
Test Duration	Hours	Hours to Days
Cost	Low	Moderate to High
Terminal to Animal	No	Yes/No
Used in Small Animals	Yes	Yes/No

Those tests routinely used in level I should be the tests that are simple to perform, inexpensive, quick and sufficiently sensitive so as to provide a good indication of damage to the renal system. These tests provide a general screen for nephrotoxicity. The tests in level II are more sensitive than those in level I, and should be better able to describe the extent of damage and aid in determining the mechanisms of damage to the system; however, they are more time-consuming, more difficult to perform and more expensive than level I tests. The evaluation of the state of development of tests, the skill necessary and the ease of performing the tests, is based on discussions with researchers and a review of their publications and other literature dealing with renal testing.

7.2 Evaluation of Renal System Tests for Application to a Screening Program

A numerical assessment of the testing techniques based on the criteria described in the previous section is shown in Table 7-3. The numerical assessment was subjectively made following discussion with researchers using these techniques and following a review of the current literature.

The advantages and disadvantages of each testing technique included in the recommended screening program are described below with a discussion of their potential application to the program. Alternative techniques are also described which could be used in place of the recommended tests to measure the same functional

TABLE 7-3

EVALUATION OF RENAL TESTING TECHNIQUES

TECHNIQUE	LEVEL OF DEVELOPMENT (ANIMALS)	LEVEL OF SKILL	SENSITIVITY IN DETECTING DAMAGE	DETECTING EXTENT OF DAMAGE	SOPHISTICATION OF EQUIPMENT	TERMINAL TO ANIMAL	TIME[1]	COST[2]	SUITABILITY FOR A SCREENING PROGRAM
• Morphologic									
- Gross Inspection	5	4	3	3	2	Yes	1	1	4
- Light Microscopy	5	4	4	4	3-4	"	3	1	4
- Electron Microscopy	4	5	4	5	4-5	"	4	3	2
• Glomerular Function Tests									
- Blood Urea Nitrogen	5	2	2	2	2	No	1	1	4
- Serum Creatinine	5	2	3	3	2	"	1	1	4
- Urinary Protein	4	3	4	4	2-3	"	2	1	4
- Inulin Clearance	4	4	3	4	3-4	"	2-3	2	4
- Creatinine Clearance	3	3	3	3	3	"	2-3	2	2
- ^{125}I-*Iothalamate and* ^{131}I-Diatrizoate Clearance	3	4	3	4	3	"	3	2	3
- Plasma Disappearance Method	3	4	3	4	4	"	3	2-3	3
• Tubular Function Tests									
- Urinary Glucose	5	2	2-3	3	2	No	2	1	4
- Glucose Transport Maximum	3	4	3	3	4	"	3-4	2	2
- Urinary Acidification	3	4	3	3	2	"	3	1-2	2
- PAH Transport Maximum	4	4	3	4	4	"	4	2	4
- Renal Cortical Slices	4	3	4-5	4	3-4	Yes	4	2-3	4
- Isolated Perfused *Tubule Segments*	2	4	2	3	4	"	4	2-3	1
- Urinary Concentrating Ability									
Specific Gravity	5	2	4	3	2	No	1	1	4
Osmolality	4	2	4	3	2	"	1	1	4
- Urinary Diluting Ability	2	2	2	2	2	"	1	1-2	2
- Microscopy of Urinary Sediment	4	4	4	4	2	"	2	1	4

TABLE 7-3

EVALUATION OF RENAL TESTING TECHNIQUES -(Concluded)

TECHNIQUE	LEVEL OF DEVELOPMENT (ANIMALS)	LEVEL OF SKILL	SENSITIVITY IN DETECTING DAMAGE	DETECTING EXTENT OF DAMAGE	SOPHISTICATION OF EQUIPMENT	TERMINAL TO ANIMAL	TIME[1]	COST[2]	SUITABILITY FOR A SCREENING PROGRAM
• Renal Hemodynamics									
- PAH Clearance	5	3	3	4	3	No	2	2	4
- Iodohippurate Clearance	4	3	3	4	3	"	2	2	3
- Iodopyracet Clearance	4	3	3	4	3	"	2	2	3
- Krypton-85 Washout	3	4	4	4	4	"	3	2-3	3
- Xenon-133 Washout	2	4	4	4	4	"	3	2-3	2
- Hydrogen Washout	2	4	2	2	4	Yes	2	2-3	3
- Krypton-85 Autoradiography	3	4	4	4	4-5	"	3	3	2
- Radioactive Microsphere Distribution	2	4	4	4	4-5	"	3-4	3	3
• Biochemical Indicators									
- beta-Glycosidase	5	2	4	3	2	No	1	1	4
- beta-Glucoronidase	5	2	4	3	2	"	1	1	4
- Alkaline Phosphatase (AlP)	5	2	4	3	2	"	1	1	4
- Lactic Acid Dehydrogenase (LDH)	5	2	3	2	2	"	1	1	3
- Acid Phosphatase (AP)	4	2	2-3	2	2	"	1	1	3
- Glutami. Oxaloacetic Transminase (GOT)	5	2	3	2	2	"	1	1	2
- Cholinesterase (CHE)	4	2	3	3	2	"	1	1	2
- Isocitrate Dehydrogenase ICDH	4	2	3	3	2	"	1	1	2

Scale: 1=poor or very low; 2=fair or low; 3=good or medium; 4=very good or high; 5=excellent or very high.

1. Time Scale
   1 = 1 hour
   2 = 1-4 hours
   3 = 1 day
   4 = 2-4 days
   5 = 1-3 weeks

2. Cost Scale
   1 = $100
   2 = $500
   3 = $1,000
   4 = $5,000
   5 = $5,000

parameters. Table 7-4 lists the tests in each level that are recommended for a tiered short-term screening program.

7.2.1 Level I Tests

Blood Urea Nitrogen (BUN)

Urea is the primary end product of nitrogen metabolism in mammals and is excreted primarily as a result of glomerular filtration. Decreased glomerular filtration as a consequence of damage will therefore increase the blood urea concentration. The methods used to measure blood urea levels require only small quantities of blood and are relatively simple to perform. The range of normal BUN values is large, so BUN is a poor indicator of early damage (i.e., damage must be severe before BUN is outside of normal ranges). Furthermore, blood urea concentrations are affected by a number of factors other than glomerular damage. BUN has been used extensively in the past and it does provide a general screen for glomerular damage even though a number of factors can affect BUN.

Serum Creatinine

Creatinine is the end product of creatine metabolism in muscle tissue. It is released and enters the plasma at a relatively constant rate where it is filtered in the glomerulus. Consequently, serum creatinine levels are inversely proportional to the glomerular filtration rate. Serum creatinine levels are easily measured in small quantities of blood. There are some daily fluctuations in creatinine levels which limit the ability to detect early glomerular

TABLE 7-4

TESTS RECOMMENDED FOR A TIERED SCREENING PROGRAM

Level I
Blood Tests
Blood Urea Nitrogen (BUN)
Serum Creatine
Urine Tests
Glucose
Protein
Specific Gravity or Osmolality
Microscopy of Urinary Sediment
Enzymes
β-glucosidase
β-glucuronidase
Alkaline phosphatase
Morphology
Gross Examination and Light Microscopy

Level II
Inulin Clearance or Plasma Disappearance of ^{125}I-Iothalamate and ^{131}I-Diatrizoate
p-Aminohippurate (PAH) Clearance or Plasma Disappearance of either ^{125}I or ^{131}I-labeled Iodohippurate and Iodopyracet
PAH Transport Maximum
Urinary Acidification
In Vitro Cortical Slices
^{25}Kr Washout and Autoradiography or Differential Isotope Microspheres
Gross Examination, Light and Electron Microscopy

damage; however, serum creatinine levels are more stable and are less effected by factors unrelated to damage when compared to blood urea nitrogen. For this reason, serum creatinine is considered more sensitive in detecting impairment of glomerular filtration than BUN. Both serum creatinine and BUN determinations are recommended for inclusion in level I of the short-term screening program.

Urinary Glucose

Glucose is readily filtered at the glomerulus and is nearly completely reabsorbed by the proximal tubules. Presence of glucose in the urine is suggestive of damage in the proximal tubules and a decreased ability to reabsorb glucose; however, glucose can occur in the urine because of disturbances in carbohydrate metabolism and some disease states in other organs. Semiquantitative determinations can be performed in urine by using a simple test strip procedure. Thus, measurement of urinary glucose is a suitable screening technique for level I tiered testing. Because of other influencing factors, glucose in the urine is not as sensitive as other procedures in the proposed screening program; however, it can provide an indication of tubular damage.

Protein in Urine

Normally the glomerulus acts as a barrier to the passage of proteins from the plasma into the glomerular filtrate. Nonetheless, some small proteins cross the glomerular membrane, but these are reabsorbed by the proximal tubules. Therefore, protein is normally

absent in the urine of most mammals. Proteinuria indicates damage to the glomeruli; however, injury to the tubules can also cause proteinuria. High proteinuria or albuminuria is indicative of glomerular damage. Tubular damage usually results in increases in low molecular weight proteins without significant albuminuria. Several simple, semiquantitative methods are available for the measurement of protein in the urine. Since protein in the urine is a sensitive indicator of glomerular damage, it is a useful technique for screening damage to the glomerulus and therefore is included in level I.

Specific Gravity and Osmolality

The ability of the kidney to concentrate urine depends primarily on tubular integrity when the glomerular filtration rate is within normal ranges. A loss of ability to concentrate urine due to tubular damage is nonspecific in that the damage may be either in the proximal or distal segments of the tubules, or both. The two methods used to measure concentrating ability are specific gravity and osmolality, and both are simple to perform and can be done on small volumes of urine. The most widespread determination method in small animals is specific gravity and it is probably the more suitable of the two methods for use in a screening program. The information obtained by osmometry is just as reliable as specific gravity measurements and may even be somewhat superior; however, osmometers are not as common as the equipment utilized for measuring specific gravity. Osmolality

is not sufficiently superior to specific gravity to warrant the cost of purchasing osmometers when the other instrumentation is already available. Many factors can affect specific gravity and osmolality and should be considered when either of these two determinations are made. Either test will provide a useful general screen and is recommended for use in level I.

Microscopy of Urinary Sediment

Urinary sediment consists of epithelial cells, leukocytes, erythrocytes, casts and crystals, and provides information concerning anatomical integrity of the kidneys. However, it provides no information concerning renal function. Excretion of large numbers of epithelial cells and casts containing cells is a sign of the tubular degenerative processes occurring during damage. This test is simple and easy to perform, requiring small volumes of urine and a bright-field, light microscope; however, it requires an experienced technologist to make proper assessment of the types and relative numbers of elements in the urinary sediment, since even normal urine contains some sediment. Because the size of the casts and the types of epithelial cells vary from different tubular sections, examination of the sediment can provide valuable information concerning the areas and extent of damage in the tubules.

Urinary Enzymes

When tubular cells degenerate, the enzymes contained in the cells pass into the urine. Most urinary enzymes are derived from

tubular cells, so elevated urinary activity is regarded as a sensitive indicator of tubular damage. Variations in urinary enzyme activity occur in many cases before functional changes are observed because of the large reserve of functional capacity in the kidneys. Therefore, the changes in urinary enzymatic activity can provide a sensitive indicator of early renal damage. The measurement of the activities of a number of urinary enzymes is performed using standard techniques which are relatively simple, easy to perform, reproducible and inexpensive. The major disadvantage is the difficulty in collecting serial, uncontaminated urine samples from small test animals and then dialyzing and preserving the samples before analysis.

Three enzymes have been recommended for use in level I of a screening program. These are beta-glucosidase, beta-glucuronidase and alkaline phosphatase. Other enzymes could also be included based on the interests of the investigators. These enzymes were chosen because they are all sensitive indicators of tubular damage and have been the enzymes of choice in assessing tubular damage by a number of investigators in the past.

Gross Examination and Light Microscopy

Gross examination and light microscopic histopathology are often useful for verifying the results from other tests in a screening program, especially since the kidney has considerable functional reserve capacity. Early structural changes may be seen that are not

clearly associated with alterations in functional parameters. The analysis and interpretation of these observations may, at times, be difficult and subject to dispute.

7.2.2 Level II Tests

Inulin Clearance

Inulin is a polyfructose with a molecular weight of approximately 5,000, which is freely filtered at the glomerulus and is neither secreted nor reabsorbed by the tubules. Therefore, inulin clearance provides an accurate measure of glomerular filtration rate. Since inulin is hydrolyzed in the gastrointestinal tract and is poorly absorbed from subcutaneous tissue or muscle, it must be administered intravenously. Constant infusion at a rate equal to excretion is required to maintain a constant plasma level. The quantitative analysis of inulin in urine and blood samples was time-consuming and demanding before the introduction of radiolabeled inulin, which has greatly simplified the analytical procedures. Inulin clearance is included in level II of the tiered screening program because it provides an accurate determination of the glomerular filtration rate and has been used extensively in various laboratory animal species. The measurement of glomerular filtration rate could be done using either creatinine, iothalamate or diatrizoate clearance methods, or by using plasma disappearance methods with a radiolabeled substance such as ^{125} I-iothalamate. Clearance procedures employing the other substances are more simple to perform than inulin

clearance because they require only a single subcutaneous injection rather than continuous intravenous infusion; however, they have been used less than inulin clearance and are less frequently reported in the literature. Nonetheless, the investigator may wish to determine GFR using a method other than inulin clearance. The plasma disappearance methods seem promising for use in screening since they are simple, rapid, accurate and easily repeated. Some additional development is required before they can be considered standard techniques.

p-Aminohippurate Clearance

When p-aminohippurate (PAH) is introduced into the blood stream by constant infusion, it is either filtered at the glomerulus or secreted into the tubular fluid via active transport mechanisms from the peritubular capillaries. Most of the PAH in the renal plasma enters the nephron and ordinarily less than 15% of the PAH entering the kidney will remain in the renal venous blood. Therefore, the volume of blood cleared of PAH closely approximates the rate of plasma flow passing functioning nephrons and, consequently, PAH clearance is used to measure effective renal plasma flow (ERPF). PAH clearance has been used extensively in the past to determine ERPF and is currently the most appropriate method for evaluating changes in renal plasma flow. Nonetheless, other substances such as iodohippurate and iodopyracet are also effectively filtered at the glomerulus and secreted in the tubules, and thus can be used to determine ERPF.

The plasma disappearance methods using radiolabeled iodohippurate and iodopyracet can also be used to determine ERPF and even though these methods are not as well developed as the clearance techniques, they show promise for use in screening since they are simple, rapid, accurate and easily repeated. The investigator may wish to use the plasma disappearance method for radiolabeled iodohippurate or another radiolabeled substance to determine ERPF in place of PAH clearance, especially if a measurement of the Tm_{PAH} is not planned after PAH clearance measurements have been completed.

PAH Transport Maximum

When PAH is increased to high plasma concentrations, a maximum rate of PAH transport is achieved (Tm_{PAH}). The PAH transport maximum has traditionally been used as an expression of "active tubular mass" in the kidneys. When the tubules are damaged, there is a decrease in the "active tubular mass" and, subsequently, a decrease in the Tm_{PAH}. The measurement of Tm_{PAH} is included in level II of the screening program because it can be determined at the same time as PAH clearance and is useful in providing an indication of "active tubular mass." Tm_{PAH} is more difficult to determine than PAH clearance because it can be altered by such factors as extracellular fluid volume expansion. When there is uneven damage in different nephrons, the maximum rate of PAH secretion in the undamaged

nephrons adaptively increases. Consequently, Tm_{PAH} seems to be insensitive to early forms of damage. Nevertheless, Tm_{PAH} is recommended for inclusion in level II of the screening program because it can be done at the same time as PAH clearance by simply increasing the plasma concentrations of PAH and provides additional information. The use of radiolabeled PAH simplifies the analytical procedures necessary for determining PAH in blood and urine samples in both the PAH clearance and transport maximum tests.

Urinary Acidification

The kidney regulates acid-base balance by secreting hydrogen ions in the tubules and reabsorbing bicarbonate ions. The test to measure the ability of the kidney to respond to disturbances in the acid-base balance is made by measuring urinary pH after hydrogen ion secretion is stimulated by loading the system with a substance such as ammonium chloride. The urinary acidification test is included in level II of the screening program because it provides information concerning damage in the tubules and is especially useful in detecting damage in various sections of the tubules (e.g., distal tubles) when it is used with complementary tests.

In Vitro Cortical Slices

Renal cortical slices are prepared from the excised kidneys of either rats or rabbits after the animals are treated with a nephrotoxin. The inhibition of the accumulation of an actively transported substance such as PAH is then determined following incubation of the

slices in media containing that substance. The renal cortical slice technique is a very sensitive technique for detecting acute renal toxicity and therefore is recommended for inclusion in level II of the screening program. The technique has the following advantages: the investigator is able to rigidly control the composition of the ambient fluid; many external systemic factors that influence tubular excretion are eliminated, and substances can be tested that are not well tolerated in live animals. The major disadvantage is that the technique is terminal to the animals used. Furthermore, care must be taken to insure that the renal tissue slices are properly prepared.

Krypton-85 Washout

The washout techniques are used to measure regional blood flow in the kidney. A radioactive inert gas such as krypton-85 is injected into the renal artery, and the rate at which the radioactive material is washed out of the kidney is measured by external scintillation counting. The measurement of blood flow by this method is based upon the assumption that the radioactive material will equilibrate between blood and tissue in the kidney in a single passage through the capillaries. Furthermore, the rate at which the substance is removed from the kidney will vary directly with the rate of blood flow through the capillaries and, if the tissue/blood partition coefficient is known, the blood flow per unit volume of tissue can be determined. Krypton-85 is used in washout techniques because it is

lipid soluble and is highly diffusible. Because of its low solubility in blood relative to air, most krypton-85 is removed in one circulation through the lung so that the amount returning to the kidney is negligible. Using the washout technique, blood flow in regional compartments and the intrarenal distribution of blood flow can be determined. By autoradiography, the following compartments have been identified: I. the outer cortex; II. the inner cortex and outer medulla; III. the inner medulla; and IV. the perirenal and hilar fat. Redistribution and regional reductions in blood flow related to damage can be described, which provide an indication of the hemodynamic mechanisms involved in damage. For this reason, the krypton-85 washout technique is recommended for level II of the tiered screening program. This technique is most commonly performed in dogs, and the procedure has been adapted to rats. Major limitations of the technique are (a) the subjective nature of the analysis of the washout curve into its component exponentials, which should correlate with specific areas of the kidney and (b) the assumption that the partition coefficients remain constant throughout the procedure.

Krypton-85 Autoradiography

At the same time the krypton-85 washout curve is being determined, autoradiograms can be taken. These are recommended when the washout technique is used to aid in the interpretation of any abnormal curves. They are especially useful in identifying the regional compartments in damaged kidneys and the extent of damage in any one compartment.

Microsphere Techniques

The extraction and entrapment of radioactive microspheres by the glomerular capillaries can be used to determine regional blood flow and the intrarenal distribution of blood flow. The microsphere techniques provide an alternative to the washout procedure; however, they have only been used in dogs and have not yet been adapted for use in rats or other small laboratory animals. The microspheres are sufficiently small (i.e., approximately 15 in diameter) that they do not damage the renal tissue or alter blood flow to the kidney and they are completely extracted by the glomerular capillaries in one circulation. The plastic microspheres are most commonly labeled with ^{169}Yb, ^{85}Sr, ^{46}Sc or ^{141}Ce. In this technique, the animal is injected with a bolus of labeled microspheres in the left ventricle or the root of the aorta, and complete mixing occurs before the microspheres reach the renal artery, so that the quantity of microspheres entraped in the renal tissue is a function of the fraction of blood flow to that tissue. The animal is then terminated and the renal cortex is removed and sliced in sections representing the compartments of the cortex. The sections are then counted using a scintillation counter. Control values can be determined using microspheres with one label. The animal can then be exposed to a nephrotoxin and injected with a second bolus of microspheres containing a different radiolabel. The differences between the distributions of the two labels can then be used to detect alterations in blood flow

distribution induced by the toxic agent. Microsphere techniques have not as yet been used in screening substances for nephrotoxicity; however, their ability to detect changes in regional blood flow in the kidney indicates their potential usefulness in assessing nephrotoxicity. The major advantage of the microsphere technique is that it gives a more precise profile of regional flow rates than the inert-gas washout techniques. A major disadvantage is that the microsphere techniques are terminal to the animals used.

The use of gross examination and light microscopic histopathology is recommended at the conclusion of Level II of the screening program. Electron microscopy is an expensive and involved technique which should be applied only to those situations where the findings from other tests are inconclusive.

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APPENDIX A

MORPHOLOGICAL DAMAGE INDICATORS

TABLE A-1

MORPHOLOGICAL DAMAGE INDICATORS

TESTS EMPLOYED	SPECIES OF ANIMALS USED	SUBSTANCES TESTED	REFERENCES	COMMENTS
GROSS OBSERVATION, LIGHT MICROSCOPY, TRANSMISSION ELECTRON MICROSCOPY (TEM), SCANNING ELECTRON MICROSCOPY (SEM)	CATS, DOGS, GUINEA PIGS, HUMANS (ADULTS), MICE, RABBITS, RATS	METHYL MERCURIC CHLORIDE, METHYL MERCURY HYDROXIDE, MERCURIC CHLORIDE, SODIUM CHROMATE, URANYL NITRATE, ETHYLENEIMINE, DIELDRIN, LEAD ACETATE, HUMAN GLOBIN, CARBON TETRACHLORIDE, PARAQUAT, DIQUAT, CADMIUM CHLORIDE, CADMIUM METALLOTHIONEIN, PROCAINE PENICILLIN G, CHLOROFORM, FLUOROCITRATE, 2,4,5-TRICHLOROPHENOXYACETATE, RED LEAD FUMES, METHOXYFLURANE, PURONUCIN AMINONUCLEOSIDE, ATRACTYLOSIDE, CARBOXYATRACTYLOSIDE, A SERIES OF 2-SUBSTITUTED FURANS, INORGANIC FLUORIDE, ZINC, GLYCERIN, THIOPHENES, CHOLINE DEFICIENCY, SODIUM ACID PHOSPHATE, NEPHROTOXIC SERUM, URANYL NITRATE HEXAHYDRATE, GENTAMICIN, MANITOL, 6-AMINONUCLEOSIDE, SULFAPYRIDINE, POLYBROMINATED BIPHENYLS	ANDREWS, 1975; BALAZS ET AL., 1963; CARPENEDO ET AL., 1974; CATER AND PETERS, 1961; CHANG AND SPRECHER, 1976; CRAMER ET AL., 1974; DALHAMN AND FRIBERG, 1957; ELLIS ET AL., 1973a & b; EVAN AND DAIL, 1974; FOWLER, 1972, 1974a; FOWLER AND WOODS, 1977; FOWLER ET AL., 1974, 1975a & b; GANOTE ET AL., 1975; GONICK ET AL., 1975; GOYER, 1968; GRITZKA AND TRUMP, 1968; HEWITT ET AL., 1979; HIRSCH ET AL., 1971; KLEIN ET AL., 1972, 1973; KOSCHIER AND BERNDT, 1976a; KOSEK ET AL., 1974; LOCK AND ISHMAEL, 1979; MAUNSBACH, 1966; MCCORMACK ET AL., 1978; MCCREIGHT AND WITCOFSKI, 1969; MCMURTRY AND MITCHELL, 1977; MENEFEE ET AL., 1964; NORDBERG ET AL., 1975; REIMER AND JENNINGS, 1971a; REIMER ET AL., 1972; SHARRAT AND FRAZER, 1963; STONE ET AL., 1961; STRIKER ET AL., 1968; SUN ET AL., 1966; SUZUKI AND MOSTOFI, 1966, 1970; TAYLOR, 1965; WARE ET AL., 1973, 1975	GROSS OBSERVATION IS AN INSENSITIVE ESTIMATION OF NEPHROTOXICITY. LIGHT MICROSCOPIC EXAMINATION CAN REVEAL GENERAL STRUCTURAL DAMAGE. ULTRASTRUCTURAL METHODS ARE REASONABLY RAPID, ALLOW LOCATION OF CHANGES WITHIN SPECIFIC AREAS OF THE KIDNEY AND REQUIRE ONLY SMALL TISSUE SAMPLES.

APPENDIX B

GLOMERULAR FUNCTION TESTS

TABLE B-1

GLOMERULAR FUNCTION TESTS

TESTS EMPLOYED	SPECIES OF ANIMALS USED	SUBSTANCES TESTED	REFERENCES	COMMENTS
MEASUREMENT OF BLOOD UREA NITROGEN (BUN)	DOGS, HUMANS (ADULTS), MICE, RATS	6-AMINONUCLEOSIDE, BACITRACIN, CHLORHEXIDINE, CHLOROFORM, DIGLYCOALDEHYDE, GENTAMICIN SULFATE, GLYCEROL, KEPONE, MERCURIC BICHLORIDE, METHYLMERCURY, METHOXYFLUORANE, METHYL-MERCURY CHLORIDE, METHYL-MERCURY HYDROXIDE, MIREX, POLYBROMINATED BIPHENYLS, POLYCHLORINATED BIPHENYLS, SODIUM CHROMATE, SULFA-PYRIDINE, TRICHLOSEN, URANYL NITRATE	BALAZS ET AL., 1963; BARR ET AL., 1973; BERNDT, 1976a; CARPENEDO ET AL., 1974; CHEDRU ET AL., 1972; CHOW ET AL., 1977; COHEN ET AL., 1975; CROWE AND HATCH, 1977; DIBONA AND SAWIN, 1971; DIBONA ET AL., 1971; FLAMENBAUM ET AL., 1971; HEWITT ET AL., 1979; HIRSCH, 1974; KAHN AND STEIN, 1972; KASSIRER, 1971a; KAUFMAN ET AL., 1977; KEOGH ET AL., 1977; KLEIN ET AL., 1972, 1973, 1977; KLUWE AND HOOK, 1978; MAZZE ET AL., 1971; MCCORMACK ET AL., 1978; MILLER ET AL., 1950; OKEN ET AL., 1966, 1970; SINGHVI ET AL., 1978; STROO AND HOOK, 1977a; THIEL ET AL., 1967, 1970; WARE ET AL., 1974; WILSON ET AL., 1967, 1969	BUN DETERMINATIONS HAVE TRADITIONALLY BEEN USED AS INDICATORS OF GLOMERULAR DYSFUNCTION EVEN THOUGH BUN IS AFFECTED BY A NUMBER OF FACTORS. FURTHERMORE, IT IS AN INSENSITIVE INDICATOR OF EARLY GLOMERULAR DAMAGE AND WILL REMAIN WITHIN NORMAL RANGES UNTIL GLOMERULAR FUNCTION IS REDUCED BY 50% OR MORE. BUN WILL INCREASE DUE TO TUBULAR NECROSIS; DEHYDRATION, ACCELEVATED PROTEIN, DEGRADATION DUE TO TRAUMA, FEVER OR INFECTION; INGESTION OF LARGE QUANTITIES OF PROTEIN; AND BREAKDOWN OF BLOOD IN THE GASTROINTESTINAL TRACT.
MEASUREMENT OF SERUM CREATININE	CATS, HUMANS (ADULTS), RABBITS, RATS	CARBON TETRACHLORIDE, ATRACTYLOSIDE, CARBOXY-ATRACTYLOSIDE, METHYL MERCURY, CADMIUM, CADMIUM CHLORIDE, GENTAMICIN, METHYL MERCURY HYDROXIDE, METHYL MERCURIC CHLORIDE, METHOXYFLURANE INOSINI DIALDEHYDE	AXELSON AND PISCATOR, 1966; CARPENEDO ET AL., 1974; COHEN ET AL., 1975; CROWE AND HATCH, 1977; ELLIS ET AL., 1973b; KAHN AND STEIN, 1972; KAUFMAN ET AL., 1977; KEMP AND LAURSEN, 1960; KLEIN ET AL., 1973; MARTINEZ AND DOOLAN, 1960; MAZZE ET AL., 1971; RICKERS ET AL., 1978; SIROTA, 1949; WARE ET AL., 1974	SERUM CREATININE MEASUREMENTS ARE SENSITIVE INDICATORS OF GLOMERULAR DYSFUNCTION AND THEY HAVE BEEN USED EXTENSIVELY IN SMALL LABORATORY ANIMALS. SERUM CREATININE LEVELS ARE MORE SENSITIVE IN DETECTING ALTERATIONS IN GLOMERULAR FUNCTION THAN BUN. THE STANDARD ALKALINE PICRATE METHOD IS A RELATIVELY SIMPLE COLORIMETRIC PROCEDURE OF DETERMINING SERUM CREATININE LEVELS; HOWEVER OTHER CHROMOGENS PRESENT IN PLASMA CAN REACT WITH THE PICRATE REAGENT AND

TABLE B-1 (CONCLUDED)

TESTS EMPLOYED	SPECIES OF ANIMALS USED	SUBSTANCES TESTED	REFERENCES	COMMENTS
MEASUREMENT OF SERUM CREATININE (con't)				LEAD TO ANALYTICAL ERRORS. SERUM CREATININE MEASUREMENTS SHOULD BE USEFUL IN DETECTING GLOMERULAR DYSFUCTION IN A SCREENING PROGRAM.
MEASUREMENT OF PROTEIN IN THE URINE	CATS, DOGS, GUINEA PIGS, HUMANS (ADULTS), MICE, RABBITS, RATS	THYLENEIMINE, N,N'-DIACETYL-BENZINDINE, CADMIUM, CADMIUM CHLORIDE, PHENOL RED, METHYL MERCURY HYDROXIDE, MERCURIC CHLORIDE, PARAQUAT, DIQUAT, ATRACTYLOSIDE, CARBOXY-ATRACTYLOSIDE, CITRININ, SODIUM CHROMATE, URANYL NITRATE, 6-AMINONUCLEOSIDE, SULFAPYRIDINE	AXELSON AND PISCATOR, 1966; BALAZS ET AL., 1963; BERNDT, 1976a; BERNDT AND HAYES, 1977; CARPENEDO ET AL., 1974; ELLIS ET AL., 1973a & b; HARMAN, 1971; KEMP AND LAURSEN, 1960; KLEIN ET AL., 1973; LOCK AND ISHMAEL, 1979; RELMAN AND LEVINSKY, 1971; SHARRATT AND FRAZER, 1963; WARE ET AL., 1974; WILSON, 1975	PROTEIN IS NORMALLY ABSENT FROM THE URINE IN MOST MAMMALS OR IS PRESENT IN ONLY TRACE QUANTITIES IN A FEW ANIMALS (e.g., RATS). THE MEASUREMENT OF PROTEIN AND ALBUMIN IN THE URINE IS A SENSITIVE INDICATOR OF EARLY GLOMERULAR DAMAGE AND HAS BEEN USED EXTENSIVELY IN SMALL LABORATORY ANIMALS; HOWEVER THE SENSITIVITY OF THIS TEST VARIES WITH THE SEX AND SPECIES OF ANIMAL USED. SEVERAL SIMPLE, SEMIQUANTITATIVE PROCEDURES ARE AVAILABLE FOR MEASURING PROTEIN IN THE URINE; THE "DIPSTICK" METHOD IS THE MOST COMMONLY USED. THE LEVELS OF PROTEIN IN THE URINE CAN BE INFLUENCED BY DAMAGE IN OTHER ORGANS BESIDES THE KIDNEY. THE TEST WOULD BE USEFUL IN DETECTING EARLY GLOMERULAR DAMAGE IN A SCREENING PROGRAM.

TABLE B-2

MEASUREMENT OF GLOMERULAR FILTRATION RATE: PLASMA CLEARANCE PROCEDURES

TESTS EMPLOYED	SPECIES OF ANIMALS USED	SUBSTANCES TESTED	REFERENCES	COMMENTS
INULIN CLEARANCE	DOGS, HUMANS (ADULTS AND CHILDREN), RABBITS, RATS, SWINE	URANYL ACETATE, URANYL NITRATE, CADMIUM CHLORIDE, LEAD CARBONATE, SODIUM ACID PHOSPHATE, TESTOSTERONE, PROGESTERONE, ESTROGENS, POLYBROMINATED BIPHENYLS, PARAQUAT, DIQUAT, MERCURIC COMPOUNDS, MERCURIC CHLORIDE, LEAD, METHEMOGLOBIN, OCHRATOXIN A, GLOBIN, POTASSIUM DICHROMATE, CLYCEROL, BALBOCAPNINE, DOPAMINE, SALURETICS	BARENBERG ET AL., 1968; BERLYNE ET AL., 1964; BRECKENRIDGE AND METCALF-GIBSON, 1965; BRENNAN ET AL., 1977; CHEDRU ET AL., 1972; CONCANNON ET AL., 1964; COHEN ET AL., 1969; CRAMER ET AL., 1974; CUTLER AND GLATTE, 1965; DIBONA ET AL., 1971; DODGE ET AL., 1967; ELLIS ET AL., 1973a & b; FARMER ET AL., 1967; FAVRE AND WING, 1968; FLAMENBAUM ET AL., 1971; FOULKES, 1971; GIESKE AND FOULKES, 1974; GLASSER, 1961; GYRD-HANSEN, 1968; GYRD-HANSEN AND HELLEBERG, 1976; HARVEY AND MELVIN, 1965, 1966; HENRY ET AL., 1968; JAENIKE, 1969; JAMISON, 1970; JOHNSON AND KLEINMAN, 1979; LOCK, 1979; MARSH AND FRASIER, 1965; MCCORMACK ET AL., 1978; MESCHAN ET AL., 1963b; MILLER ET AL., 1950, 1952; NELP ET AL., 1964; NOMIYAMA AND FOULKES, 1968; NOMIYAMA ET AL., 1974; OESTER ET AL., 1969; OKEN ET AL., 1970; PITTS, 1974; RELMAN AND LEVINSKY, 1971; RONQUILLO ET AL., 1968; ROSENBAUM, 1970; ROSENBAUM ET AL., 1967; SCHNERMAN ET AL., 1971; SHARRATT AND FRAZER, 1963; SIGMAN ET AL., 1965; SILKALNS ET AL., 1973; SULLIVAN, 1974; SUMMERS ET AL., 1967; SUZUKI ET AL., 1975; VALEK, 1965; WILSON, 1977	INULIN CLEARANCE PROVIDES AN ACCURATE MEASURE OF GFR AND HAS TRADITIONALLY BEEN THE MOST COMMON METHOD USED FOR THE DETERMINATION OF GFR IN LABORATORY ANIMALS. THE CHEMICAL QUANTITATION OF INULIN IS TIME CONSUMING AND DEMANDING UNLESS RADIOLABELED INULIN IS USED. THERE ARE ALSO AUTOMATED METHODS AVAILABLE FOR THE DETERMINATION OF INULIN. SINCE INULIN IS HYDROLYZED IN THE GASTROINTESTINAL TRACT AND IS POORLY ABSORBED FROM SUBCUTANOUS TISSUE OR MUSCLE, IT MUST BE ADMINISTERED INTRAVENOUSLY AND CONSTANT INFUSION IS REQUIRED TO MAINTAIN CONSTANT PLASMA LEVELS. GFR MAY BE DETERMINED IN A SCREENING PROGRAM USING INULIN CLEARANCE OR ALTERNATIVE TECHNIQUES.

TABLE B-2 (CONTINUED)

TESTS EMPLOYED	SPECIES OF ANIMALS USED	SUBSTANCES TESTED	REFERENCES	COMMENTS
CREATININE CLEARANCE	CATS, DOGS, HUMANS (ADULTS AND CHILDREN), RABBITS, RATS, SWINE	MERCURIC CHLORIDE, METHYL MERCURY CHLORIDE, URANYL ACETATE, URANYL NITRATE, HYDROCHLOROTHIAZIDE, CADMIUM CHLORIDE, ETHYLENEIMINE, ZINC, POLYMYXIN, LEAD CARBONATE, TESTOSTERONE, ESTROGENS, SODIUM FERROCYANATE, ATRACTYLOSIDE, POLYTHIAZIDE, CHLOROTHIAZIDE, ACETAZOLAMIDE, FUROSEMIDE, ETHACRYNIC ACID, PROBENECID, ACTH, CORTISONE, METHOXYFLURANE, INOSINE, DIALDEHYDE, MANNITOL, CARBOXYATRACTYLOSIDE	AXELSON AND PISCATOR, 1966; BAER ET AL., 1959; BERLYNE ET AL., 1964; BRECKENRIDGE AND METCALF-GIBSON, 1965; CARPENEDO ET AL., 1974; COHEN ET AL., 1969; CROWE AND HATCH, 1977; DODGE ET AL., 1967; ELLIS ET AL., 1973a & b; FAVRE AND WING, 1968; GIESKE AND FOULKES, 1974; GLASSER, 1961; GYRD-HANSEN, 1968; HARVEY AND MALVIN, 1965, 1966; JOHNSON AND KLEINMAN, 1979; KASSIRER, 1971a; KAUFMAN ET AL., 1977; KEMP AND LAURSEN, 1960; KEOGH ET AL., 1977; KLEIN ET AL., 1973; MALVIN ET AL., 1958; MARTINEZ AND DOOLAN, 1960; MAZZE ET AL., 1971; MILLER ET AL., 1952; MOYER ET AL., 1953; NOMIYAMA AND FOULKES, 1968; PITTS, 1974; RAM ET AL., 1969; RELMAN AND LEVINSKY, 1971; RONQUILLO ET AL., 1968; ROSENBAUM, 1970; SADOWSKI ET AL., 1977; SAPIRSTEIN ET AL., 1955; SILKALNS ET AL., 1973; SINGHVI ET AL., 1978; STRIBRNA AND SCHUCK, 1975; STOKES AND TER-POGOSSIAN, 1964; VALEK, 1975; VANDER, 1963; YOUNG AND EDWARDS, 1964; WILSON, 1977	CREATININE CLEARANCE HAS BEEN USED IN THE PAST TO DETERMINE GFR; HOWEVER IN RATS AND GUINEA PIGS CREATININE IS SECRETED BY THE TUBULES AND THUS GIVES AN INACCURATE GFR VALUE FOR THESE ANIMAL SPECIES. TUBULAR SECRETION IS NOT A PROBLEM IN DOGS, RATS AND RABBITS AND CREATININE CLEARANCE HAS BEEN USED SUCCESSFULLY IN THESE ANIMALS. CREATININE CAN EITHER BE ADMINISTERED TO THE ANIMAL BY CONSTANT INFUSION OR IT MAY BE GIVEN SUBCUTANEOUSLY. THE STANDARD ALKALINE PICRATE METHOD OF ANALYSIS IS THE MOST COMMON ANALYTIC METHOD USED; HOWEVER SINCE PLASMA BACKGROUND CHROMOGENS REACT WITH THE PICRATE REAGENT ERROR IS INTRODUCED IN THE ANALYSIS. CREATININE CLEARANCE IS NOT USED AS MUCH FOR THE DETERMINATION OF GFR AS INULIN CLEARANCE OR OTHER METHODS.

TABLE B-2 (CONTINUED)

TESTS EMPLOYED	SPECIES OF ANIMALS USED	SUBSTANCES TESTED	REFERENCES	COMMENTS
^{125}I-IOTHALAMATE OR ^{131}I-DIATRIZOATE CLEARANCE	DOGS, HUMANS (ADULTS AND CHILDREN), RATS	CADMIUM CHLORIDE	BRYAN ET AL., 1972; COHEN ET AL., 1969; FARMER ET AL., 1967; GONICK ET AL., 1975; NELP ET AL., 1964; OESTER ET AL., 1969; PIHL, 1974; PIHL AND NOSSLIN, 1974; POWERS ET AL., 1977; RAM ET AL., 1969; SILKALNS ET AL., 1973; STOKES AND TER-POGOSSIAN, 1964; WILSON, 1977	^{125}I-IOTHALAMATE (IOT) CLEARANCE AGREES WELL WITH INULIN CLEARANCE AND IS COMMONLY USED IN DOGS AND RATS. ^{131}I-DIATRIZOATE (DTZ) CLEARANCE IS MAINLY USED IN DOGS AND HAS NOT BEEN REPORTED FREQUENTLY IN THE LITERATURE. BOTH SUBSTANCES ARE ADMINISTERED BY INTRAVENOUS INFUSION OR SUBCUTANEOUS INJECTION AND, WITH THE RADIOLABEL, THE ANALYTICAL PROCEDURES FOR THE DETERMINATION OF IOT AND DTZ IN PLASMA AND URINE ARE SIMPLE AND RAPID. THESE TWO SUBSTANCES CAN BE USED AS ALTERNATIVES TO INULIN FOR THE DETERMINATION OF GFR IN A SCREENING PROGRAM.
UREA CLEARANCE	HUMANS (ADULTS), RATS, SWINE	ACETAZOLAMIDE, ATRACTYLOSIDE, CARBONTETRACHLORIDE, CARBOXYATRACTYLOSIDE, CHLOROTHIAZIDE, DIQUAT, ETHACRYNIC ACID, FUROSEMIDE, GENTAMICIN, METHOXYFLUORANE, PARAQUAT, POLYTHIAZIDE	BARR ET AL., 1973; BERLYNE ET AL., 1964; CARPENEDO ET AL., 1974; CROWE AND HATCH, 1977; GYRD-HANSEN, 1968; LOCK, 1979; SIROTA, 1969; STRIBRNA AND SCHUCK, 1975	A VARIABLE AMOUNT OF UREA CAN BE PASSIVELY REABSORBED FROM THE TUBULAR FLUID, SO MEASUREMENT OF UREA CLEARANCE MAY UNDERESTIMATE GFR. THE MEASUREMENT IS MARKEDLY INFLUENCED BY SUCH FACTORS AS NITROGEN METABOLISM AND RATE OF URINE FLOW.

TABLE B-2 (CONCLUDED)

TESTS EMPLOYED	SPECIES OF ANIMALS USED	SUBSTANCES TESTED	REFERENCES	COMMENTS
OTHER SUBSTANCES CLEARANCE (VITAMIN B_{12}, ^{3}H MANNITOL, ^{51}Cr EDETIC ACID, ^{14}C NADOLOL)	DOGS, HUMANS (ADULTS AND CHILDREN), RATS	MERCURIC CHLORIDE, URANYL NITRATE	BREAKENRIDGE AND METCALF-GIBSON, 1965; CUTLER AND GLATTE, 1965; DIBONA ET AL., 1971; FAVRE AND WING, 1968; HOUCK, 1949; MALVIN ET AL., 1958; MESCHAN ET AL., 1963a; NELP ET AL., 1964; OESTER ET AL., 1969; SAPIRSTEIN ET AL., 1955; SINGHVI ET AL., 1978; SUMMERS ET AL., 1967.	

TABLE B-3

MEASUREMENT OF GLOMERULAR FILTRATION RATE: PLASMA DISAPPEARANCE PROCEDURES

TESTS EMPLOYED	SPECIES OF ANIMALS USED	SUBSTANCES TESTED	REFENRENCES	COMMENTS
^{125}I-IOTHALAMATE DISAPPEARANCE	DOGS, HUMANS (ADULTS AND CHILDREN), MICE, RATS	PARAQUAT	BRYAN ET AL., 1972; COHEN ET AL., 1971; ECKER ET AL., 1975b; PIHL, 1974; POWERS ET AL., 1977; RAM ET AL., 1969; SILKALNS ET AL., 1973; STOKES AND TER-POGOSSIAN, 1964; TRUNINGER ET AL., 1968	THE PLASMA DISAPPEARANCE METHODS DO NOT REQUIRE THE COLLECTION OF ACCURATELY TIMED URINE SAMPLES, BLADDER CATHETERIZATION OR INTRA-VENOUS INFUSION OF THE DIAGNOSTIC AGENT THE WAY CLEARANCE PROCEDURES DO SO THEY ARE MUCH SIMIPLER AND QUICKER TO PERFORM THAN THE CLEARANCE PROCEDURES. THEY HAVE ALSO NOT BEEN USED AS OFTEN AS THE CLEARANCE TECHNIQUES AND THEREFORE ARE NOT AS WELL DEVELOPED. THE PLASMA DISAPPEARANCE METHODS SEEM PROMISING FOR SCREENING SINCE THEY ARE SIMPLE, RAPID, ACCURATE AND THEY ARE EASILY REPEATED IN SERIAL DETERMINA-TIONS.
^{131}I-DIATRIZOATE DISAPPEARANCE	HUMANS (ADULTS)		FARMER ET AL., 1967	

APPENDIX C

TUBULAR FUNCTION TESTS

TABLE C-1

TUBULAR FUNCTION TESTS: REABSORPTIVE TESTS

TESTS EMPLOYED	SPECIES OF ANIMALS USED	SUBSTANCES TESTED	REFERENCES	COMMENTS
MEASUREMENT OF GLUCOSE IN THE URINE	HUMANS (ADULTS), MICE, RABBITS, RATS	SODIUM CHROMATE, URANYL NITRATE, MERCURIC CHLORIDE, URANYL ACETATE, CHLORINATED METHANE, ETHANE AND ETHYLENE DERIVATIVES, PARAQUAT, DIQUAT, CADMIUM CHLORIDE	BALAZS ET AL., 1963; BERNDT, 1976a; CROWE AND HATCH, 1977; DIEZI AND BIOLLAZ, 1979; KASSIRER, 1971b; LOCK AND ISHMAEL, 1979; NOMIYAMA ET AL., 1973, 1974; PLAA AND LARSON, 1965; WILSON, 1975	GLUCOSE DOES NOT NORMALLY APPEAR IN THE URINE AND WHEN IT DOES, IT IS SUGGESTIVE OF TUBULAR DYSFUNCTION. OTHER FACTORS SUCH AS DISTURBANCES IN CARBOHYDRATE METABOLISM CAN ALSO RESULT IN GLUCOSURIA. FOR THIS REASON, THE MEASUREMENT OF GLUCOSE IN URINE IS NOT CONSIDERED AS SENSITIVE AN INDICATOR OF TUBULAR DAMAGE AS OTHER TESTS. BECAUSE IT IS EASILY DETERMINED SEMI-QUANTITATIVELY BY "DIPSTICK" METHODS AND DOES PROVIDE AN INDICATION OF TUBULAR DAMAGE, IT SHOULD BE USEFUL IN A GENERAL SCREEN FOR NEPHRO-TOXICITY.
MEASUREMENT OF TRANSPORT MAXIMUM FOR GLUCOSE (TM_G)	DOGS, HUMANS (ADULTS), RABBITS	BACITRACIN, CADMIUM, MERCURY, ZINC, URANYL ACETATE	MILLER ET AL., 1950; NOMIYAMA AND FOULKES, 1968; VANDER, 1963	THE TRANSPORT MAXIMUM FOR GLUCOSE (TM_G) IS DETERMINED BY LOADING THE TUBULAR CELLS WITH MORE SUBSTANCE THAN CAN BE REABSORBED. THIS IS ACCOMPLISHED BY A CONSTANT INTRAVENOUS INFUSION OF GLUCOSE AND THEN URINE SAMPLES ARE COLLECTED USING A CATHETER. THE GFR MUST BE DETERMINED TO CALCULATE THE TM_G. THE TM_G IS NOT ESPECIALLY SENSITIVE IN DETECTING TUBULAR DAMAGE AND IT IS AFFECTED BY A NUMBER OF FACTORS. FOR THESE REASONS, THE MEASUREMENT OF TM_G WOULD HAVE ONLY LIMITED APPLICATION.

TABLE C-2

TUBULAR FUNCTION TESTS: SECRETORY TESTS

TESTS EMPLOYED	SPECIES OF ANIMALS USED	SUBSTANCES TESTED	REFERENCES	COMMENTS
MEASUREMENT OF URINARY ACIDIFICATION	HUMANS (ADULTS), RATS	PITRESSIN, AMPHOTERICIN B	DIEZI AND BIOLLAZ, 1979; EDWARDS ET AL., 1971; GOUGE AND ANDRIOLE, 1971; KASSIRER, 1971b; MORRIS, 1969; RECTOR, 1973; RODRIGUEZ-SORIANO AND EDELMANN, 1969	THE URINARY ACIDIFICATION ABILITY OF THE KIDNEYS IS MEASURED BY STIMULATING PROTON SECRETIONS. THIS IS ACCOMPLISHED BY ADMINISTERING A SALT SUCH AS AMMONIUM CHLORIDE TO THE EXPERIMENTAL ANIMAL AND MEASURING THE pH OF THE URINE. THIS TEST IS USED IN DETECTING RENAL TUBULAR ACIDOSIS AND IS ESPECIALLY USEFUL IN DETECTING DAMAGE IN VARIOUS SECTIONS OF THE TUBULES (e.g. DISTAL TUBULES) WHEN IT IS USED WITH COMPLEMENTARY TESTS.
MEASUREMENT OF TRANSPORT MAXIMUM FOR PAH (TM_{PAH})	DOGS, HUMANS (ADULTS), RABBITS, RATS	BACITRACIN, CARBON TETRACHLORIDE, CHOLINE DEFICIENCY, CADMIUM, MERCURY, ZINC NEPHROTOXIC SERUM, SODIUM ACID PHOSPHATE, URANYL ACETATE, URANYL NITRATE, CADMIUM CHLORIDE	MILLER ET AL., 1950; NIMIYAMA AND FOULKES, 1968; NOMIYAMA ET AL., 1971; SHARRATT AND FRAZER, 1963; SINGHVI ET AL., 1978; SIROTA, 1949; VANDER, 1962, 1963	THE TRANSPORT MAXIMUM OF PAH (TmPAH) IS OCCASIONALLY USED TO ESTIMATE THE AMOUNT OF "ACTIVE RENAL TUBULAR MASS" IN THE KIDNEYS. TmPAH SEEMS TO BE INSENSITIVE TO EARLY DAMAGE AND IS ALTERED BY VARIOUS EXTRARENAL FACTORS. TmPAH IS USEFUL IN DETERMINING THE EXTENT OF TUBULAR DAMAGE AND MAY HAVE UTILITY IN A SCREENING PROGRAM. THE ANALYTICAL PROCEDURES FOR PAH ARE DIFFICULT, HOWEVER THE USE OF RADIOLABELED PAH SIMPLIFIES THE PROCEDURES.

TABLE C-3

TUBULAR FUNCTION TESTS: IN VITRO CORTICAL SLICE TECHNIQUES

TESTS EMPLOYED	SPECIES OF ANIMALS USED	SUBSTANCES TESTED	REFERENCES	COMMENTS
ACCUMULATION OF PAH, NMN, TEA, PSP, ALB, OTHER ACIDIC AND BASIC SUBSTANCES (TRANSPORT MECHANISMS)	MICE, RATS, GUINEA PIGS, RABBITS, CATS, DOGS	MERCURIC CHLORIDE, METHYL MERCURIC CHLORIDE, URANYL NITRATE, LEAD ACETATE, POTASSIUM CHROMATE, POTASSIUM DICHROMATE, GLYCEROL, GENTAMICIN SULFATE, CARBON TETRACHLORIDE, PARAQUAT, DIQUAT, 1,1,2-TRICHLOROETHANE, 2,4-DICHLOROPHENOXYACETIC ACID, 2,4,5-TRICHLOROPHENOXY-ACETIC ACID, CHROMIUM, *OCHRATOXIN A, CITRINIA*, MIREX, KEPONE, PROCAINE PENICILLIN G, POLYBROMINATED BIPHENYLS, TRICHLOSAN, CHLORCHEXIDINE, POTASSIUM CHLORIDE, LEAD CHLORIDE, TRICHLOROETHYLENE, TETRACHLOROETHYLENE, POLY-CHLORINATED BIPHENYL, URANYL ACETATE, FOLIC ACID, NEOMYCIN, CHLOROFORM	BERNDT, 1976c, 2977; BERNDT AND HAYES, 1977; BERNDT AND KOSCHIER, 1973; CHOW ET AL., 1977; CROSS AND TAGGART, 1950; ECKER ET AL., 1975a & b; FARAH ET AL., 1959; GANOTE ET A.,.1975; HEWITT ET AL., 1979; HIRSCH, 1972; 1973a & b, 1974, 1976; HIRSCH AND HOOK: 1970; HIRSCH ET AL., 1971; HOOK AND MUNRO, 1968; HOOK ET AL., 1974; KLUWE AND HOOK, 1978; KLUWE ET AL., 1979; KOSCHIER AND BERNDT, 1976a & b, 1977a and b; KOSCHIER ET AL., 1978; LOCK AND ISHMAEL, 1979; MCCORMACK ET AL. 1978; REIMER AND JENNINGS, 1971a & b, STROO AND HOOK, 1977a & b; SUZUKI ET AL., 1975; TUNE ET AL., 1969; WATROUS AND PLAA, 1972a & b; WELCH AND BUSH, 1970; ZORZOLI AND LI, 1967.	THE IN VITRO RENAL CORTICAL SLICE TECHNIQUE IS A VERY SENSITIVE TECHNIQUE FOR ACCESSING ACUTE TOXICITY AND SHOULD HAVE CONSIDERABLE APPLICATION IN A SCREENING PROGRAM. IT HAS THE FOLLOWING ADVANTAGES: THE CHEMICAL COMPOSITION OF THE AMBIENT FLUID CAN BE RIGIDLY CONTROLLED; EXTERNAL FACTORS THAT SYSTEMICALLY ALTER TUBULAR EXCRETION CAN BE ELIMINATED; THE TECHNIQUE PERMITS EXAMINATION OF METABOLIC INHIBITORS THAT CAN NOT BE TOLERATED IN ANIMALS AND MORE OBSERVATIONS CAN BE MADE WITH THIS TECHNIQUE THAN COULD BE MADE IN LIVE ANIMALS. THE MAJOR DISADVANTAGE IS THAT THE TECHNIQUE IS TERMINAL TO THE ANIMAL. FURTHERMORE, CARE MUST BE TAKEN TO INSURE THAT THE RENAL TISSUE SLICES ARE PROPERLY PREPARED.

TABLE C-3 (CONCLUDED)

TESTS EMPLOYED	SPECIES OF ANIMALS USED	SUBSTANCES TESTED	REFERENCES	COMMENTS
AMMONIA AND GLUCOSE SYNTHESIS AND O_2 CONSUMPTION	MICE, RATS	MERCURIC CHLORIDE, METHYL MERCURIC CHLORIDE, POTASSIUM CHROMATE, POTASSIUM DICHROMATE, LEAD ACETATE, GLYCEROL, GENTAMICIN SULFATE, 2,4,5-TRICHLOROPHENOXYACETIC ACID, PARAQUAT, DIQUAT, POLYBROMINATED BIPHENYLS, LEAD CHLORIDE, ACTINOMYCIN D	CROSS AND TAGGART, 1950; ECKER ET AL., 1975a; GANOTE ET AL., 1975a; HIRSCH, 1973a & b, 1974; KOSCHIER AND BERNDT, 1976a, 1977a; LOCK AND ISHMAEL, 1979; MCCORMACK ET AL., 1978; REIMER AND JENNINGS, 1971b, RUTMAN ET AL., 1965; STROO AND HOOK, 1977b; ZORZOLI AND LI, 1967	
ELECTROLYTES UPTAKE	RATS	CITRININ, MERCURIC CHLORIDE	BERNDT AND HAYES, 1977; GANOTE ET AL., 1975	

TABLE C-4

TUBULAR FUNCTION TESTS: ISOLATED PERFUSED RENAL TUBULE PREPARATIONS

TESTS EMPLOYED	SPECIES OF ANIMALS USED	SUBSTANCES TESTED	REFERENCES	COMMENTS
PAH, GLUCOSE, WATER AND ELECTROYLTE SECRETION AND ABSORPTION RATES, ELECTRICAL RESISTANCE AND CONDUCTANCE, MICROSCOPIC STRUCTURAL STUDIES	RATS, RABBITS	PAH, GLUCOSE, VARIOUS *PERFUSION FLUIDS AND* ELECTROLYTES	BURG AND ORLOFF, 1973; GIEBISCH *ET AL., 1964; TUNE ET AL., 1969*	THE PERFUSION OF ISOLATED *TUBULE SEGMENTS IS USEFUL* AS A RESEARCH TECHNIQUE FOR EXAMINING TRANSPORT MECHANISMS IN VARIOUS SECTIONS OF THE RENAL TUBULES. IT IS A RESEARCH TECHNIQUE AND WOULD NOT BE USEFUL IN SCREENING NEPHROTOXIC SUBSTANCES

TABLE C-5

TUBULAR FUNCTION TESTS: URINARY CONCENTRATING AND DILUTING ABILITY

TESTS EMPLOYED	SPECIES OF ANIMALS USED	SUBSTANCES TESTED	REFERENCES	COMMENTS
SPECIFIC GRAVITY AND OSMOLALITY	HUMANS (ADULTS), RABBITS, RATS, SYRIAN GOLDEN HAMSTERS, SWINE	MERCURIC CHLORIDE, METHYL-MERCURIC CHLORIDE, METHOXY-ETHYL MERCURY, AMYL CINNAMIC ALDEHYDE, URANYL ACETATE, URANYL NITRATE, LEAD CARBONATE, GENTAMICIN, METHOXYFLURANE, CITRININ, SODIUM CHROMATE, POTASSIUM DICHROMATE, 6-AMINONUCLEO-SIDE, AMPHOTERICIN B, GLYCEROL, HUMAN GLOBIN, GLUCOSE, 2-CHLORO-4-NITRO-BENZAMIDE, 3,5-DINITRO-BENZAMIDE, 3,5-DINITRO-O-TOLUAMIDE, HALOTHANE, MANNITOL, UREA, AMMONIUM CHLORIDE	ARTHAUD AND LOOMIS, 1978; BALAZS ET AL., 1963; BARR ET AL., 1973; BERNDT AND HAYES, 1977; BOWMAN AND FOULKES, 1970; CARPANINI ET AL., 1973; COHEN ET AL., 1975; CROWE AND HATCH, 1977; DELEON ET AL., 1960; DIEZI AND BIOLLAZ, 1979; ELLIS ET AL., 1973a; FLANIGAN AND OKEN, 1965; FOULKES AND HAMMOND, 1975; GOTTSCHALK AND MYLLE, 1959; GYRD-HANSEN AND HELLEBERG, 1976; JOHNSON AND KLEINMAN, 1979; JONES ET AL., 1965; KLEIN ET AL., 1973; KOSEK ET AL., 1974; MAZZE ET AL., 1971; MCCURDY ET AL., 1968; MENEFEE ET AL., 1964; MERONEY AND RUBINI, 1959; OKEN ET AL., 1966; PATEL ET AL., 1975; RELMAN AND LEVINSKY, 1971; SADOWSKY ET AL., 1977; SHARRATT AND FRAZER, 1963; STROO AND HOOK, 1977b; TESCHAN AND MASON, 1963; THIEL ET AL., 1967; WILLIAMS ET AL., 1969; WILSON, 1975; WINDHAGER AND GIEBISCH, 1961	SPECIFIC GRAVITY AND OSMOLALITY DETERMINATIONS ARE USED TO MEASURE THE KIDNEYS' URINE CONCENTRATING ABILITY. BOTH MEASUREMENTS ARE SIMPLE TO PERFORM AND CAN BE MADE WITH ONLY SMALL VOLUMES OF URINE (0.5 ml). MANY FACTORS OTHER THAN TUBULAR DAMAGE CAN AFFECT SPECIFIC GRAVITY (DIARRHEA, FEVER, PROTEINURIA, GLYCOSURIA) AND OSMOLALITY (e.g., SYSTEMIC HYPERTENSION, LOW PROTEIN DIET, THE USE OF DIURETICS, CORTICOSTEROIDS AND OTHER DRUGS). EITHER TEST SHOULD BE USEFUL IN A GENERAL SCREENING PROGRAM IN DETECTING DAMAGE TO THE CONCENTRATING MECHANISMS OF THE KIDNEYS.

TABLE C-6

TUBULAR FUNCTION TESTS: GENERAL TUBULAR DAMAGE

TESTS EMPLOYED	SPECIES OF ANIMALS USED	SUBSTANCES TESTED	REFERENCES	COMMENTS
MICROSCOPIC ANALYSIS OF URINE SEDIMENT (i.e., CELLS, CASTS, CRYSTALS)	HUMANS (ADULTS), RATS	SODIUM CHROMATE, URANYL NITRATE, MERCURIC CHLORIDE, PARAQUAT, DIQUAT, SODIUM ACID PHOSPHATE, ANTI-RAT-KIDNEY SERUM	BALAZS ET AL., 1963; DIEZI AND BIOLLAZ, 1979; LOCK AND ISHMAEL, 1979; PRESCOTT AND ANSARI, 1969; RELMAN AND LEVINSKY, 1971; SHARRATT AND FRAZER, 1963; WILSON, 1975	URINARY SEDIMENT CONSISTS OF EPITHELIAL CELLS, LEUKOCYTES, ERYTHROCYTES, CASTS AND CRYSTALS AND PROVIDES INFORMATION CONCERNING ANATOMICAL INTEGRITY; HOWEVER, IT PROVIDES NO INFORMATION CONCERNING RENAL FUNCTION. SINCE NORMAL URINE SAMPLES CONTAIN SOME SEDIMENT AN EXPERIENCED TECHNOLOGIST IS NECESSARY TO MAKE PROPER ASSESSMENT OF THE TYPES AND RELATIVE NUMBERS OF ELEMENTS IN THE URINE SAMPLE. THE TEST IS SIMPLE TO PERFORM USING AN ORDINARY BRIGHT-FIELD, LIGHT MICROSCOPE. PROPER ASSESSMENT OF ABNORMAL SEDIMENT SAMPLES CAN PROVIDE VALUABLE INFORMATION CONCERNING THE AREAS AND EXTENT OF DAMAGE IN THE TUBULES AND THIS TEST SHOULD BE INCLUDED IN A ROUTINE SCREENING PROGRAM FOR NEPHROTOXICITY.

APPENDIX D

MEASUREMENT OF RENAL HEMODYNAMICS

TABLE D-1

MEASUREMENTS OF RENAL HEMODYNAMICS: CLEARANCE PROCEDURES

TESTS EMPLOYED	SPECIES OF ANIMALS USED	SUBSTANCES TESTED	REFERENCES	COMMENTS
PARA-AMINOHIPPURIC (PAH) ACID CLEARANCE	DOGS, HUMANS (ADULTS AND CHILDREN), RABBITS, RATS	ACETAZOLEAMIDE, BULBOCAPNINE, HYDROCHLOROTHIAZIDE, FUROSEMIDE, TRIAMTERENE, POLYBROMINATED BIPHENYLS	BINNION AND CUMMINGS, 1967; BLAUFOX AND MERRILL, 1966; BLAUFOX ET AL., 1963, 1967a & b; BRENNAN ET AL., 1977; CUTLER AND GLATTE, 1965; FARMER ET AL., 1967; FOULKES AND MILLER, 1959; GOTT ET AL., 1962; GYRD-HANSEN, 1968; HOUCK, 1949; JOHNSON AND KLEINMAN, 1979; MALVIN ET AL., 1958; MCCORMACK ET AL., 1978; MOYER ET AL., 1953; OESTER ET AL., 1969; POWERS ET AL., 1977; SILKALNS ET AL., 1973; SLOTKOFF ET AL., 1971; STOKES AND TER-POGOSSIAN, 1964; SUMMERS ET AL., 1967; TRUNIGER ET AL., 1968	PAH CLEARANCE HAS BEEN THE MOST EXTENSIVELY USED PROCEDURE IN THE PAST FOR THE DETERMINATION OF EFFECTIVE RENAL PLASMA FLOW (ERPF) AND IS STILL THE MOST APPROPRIATE METHOD FOR EVALUATING CHANGES IN RENAL PLASMA FLOW. PAH IS ADMINISTERED BY CONSTANT INFUSION AND THE TECHNIQUE REQUIRES THE COLLECTION OF SERIAL URINE AND BLOOD SAMPLES. THE COLORIMETRIC PROCEDURE FOR THE DETERMINATION OF PAH IS TEDIOUS AND TIME CONSUMING AND HAS LARGELY BEEN REPLACED BY METHODS USING RADIOLABELED PAH.
^{131}I- OR ^{123}I-ORTHOIODOHIPPURATE CLEARANCE (OIH)	DOGS, HUMANS		CUTLER AND GLATTE, 1965; MESCHAN ET AL., 1963a & b; PIHL, 1974; PRITCHARD ET AL., 1965; SUMMERS ET AL., 1967	BOTH OIH AND DIODRAST ARE COMPLETELY FILTERED AT THE GLOMERULUS AND EFFECTIVELY SECRETED BY THE TUBULES AND THEREFORE ARE SUITABLE FOR MEASURING ERPF. SINCE THE RADIOLABELED OIH AND DIODRAST ARE MOST COMMONLY USED, THE ANALYSIS OF BLOOD AND URINE SAMPLES IS SIMPLE, RAPID AND ACCURATELY ACCOMPLISHED BY SCINTILLATION COUNTING. BOTH MATERIALS CAN BE USED AS AN ALTERNATIVE TO PAH FOR DETERMINING ERPF IN A SCREENING PROGRAM.
^{131}I-IODOPYRACET CLEARANCE (DIODRAST)	DOGS		OESTER ET AL., 1969	

TABLE D-2

MEASUREMENTS OF RENAL HEMODYNAMICS: DISAPPEARANCE PROCEDURES

TESTS EMPLOYED	SPECIES OF ANIMALS USED	SUBSTANCES TESTED	REFERENCES	COMMENTS
^{125}I- OR ^{131}I-ORTHOIODO-HIPPURATE PLASMA DISAPPEARANCE	DOGS, HUMANS (CHILDREN AND ADULTS), RATS	INOSINE DIALDEHYDE (DIGYCOALDEHYDE)	BINNION AND CUMMINGS, 1967; BLAUFOX AND MERRILL, 1966 BLAUFOX ET AL., 1963, 1967a & b; COHEN ET AL., 1971; CUTLER AND GLATTE, 1965; FARMER ET AL., 1967; GOTT ET AL., 1962; KAUFMAN ET AL., 1977; PIHL, 1974; PIHL AND NOSSLIN, 1974; POWERS ET AL., 1977; PRITCHARD ET AL., 1965; RAZZAK ET AL., 1965, 1968; SILKALNS ET AL. 1973; STOKES AND TER-POGOSSIAN, 1964; SUMMERS ET AL., 1967; TRUNIGER ET AL., 1968; VITYE AND LEBEL, 1969.	THE PLASMA DISAPPEARANCE METHODS DO NOT REQUIRE THE COLLECTION OF ACCURATELY TIMED URINE SAMPLES, BLADDER CATHETERIZATION OR INTRA-VENOUS INFUSION OF THE DIAGNOSTIC AGENT AS IS THE CASE WITH CLEARANCE PROCEDURES; THUS ARE SIMPLER TO PERFORM, MORE RAPID, ACCURATE AND ARE EASILY REPEATED. THE DISADVANTAGE IS THAT THEY ARE NOT AS WELL DEVELOPED AS THE CLEARANCE TECHNIQUES. WITH FURTHER DEVELOPMENT AND STANDARDIZATION THEY SHOULD HAVE CONSIDERABLE APPLICATION IN A SCREENING PROGRAM.

TABLE D-3

MEASUREMENTS OF RENAL HEMODYNAMICS: REGIONAL BLOOD FLOW AND INTRARENAL DISTRIBUTION OF BLOOD FLOW

TESTS EMPLOYED	SPECIES OF ANIMALS USED	SUBSTANCES TESTED	REFERENCES	COMMENTS
KRYPTON-85 (^{85}Kr) AND XENON-133 (^{133}Xe) WASHOUT	DOGS, HUMANS (ADULTS) RATS	GLYCEROL, CARBON TETRACHLORIDE, MERCURY BICHLORIDE, SODIUM COLISTIMETHATE, INOSINE DIALDEHYDE (DIGLYCOALDEHYDE)	AYER ET AL., 1971; CARRIERE, 1970; HOLLENBERG ET AL., 1970; KAUFMANN ET AL., 1977; PITTS, 1974; THORBURN ET AL., 1963	THE RADIOACTIVE INERT GAS IS DISSOLVED IN SALINE AND INJECTED IN THE RENAL ARTERY AND THEN THE RATE THAT THE GAS IS WASHED OUT OF THE KIDNEY IS MEASURED BY EXTERNAL SCINTILLATION DETECTION. THE DISAPPEARANCE OF THE RADIOACTIVITY IS A COMPLEX FUNCTION OF TIME AND IS DESCRIBED BY A SERIES OF EXPONENTIALS, EACH ASSOCIATED WITH BLOOD FLOW THROUGH LOCALIZED REGIONS OF THE KIDNEYS. HOWEVER, THERE IS DIFFICULTY IN ASSOCIATING THE DERIVED EXPONENTIALS WITH SPECIFIC COMPONENTS OF THE KIDNEY. ANOTHER PROBLEM IS THAT THE PARTITION COEFFICIENTS ARE ASSUMED TO REMAIN CONSTANT THROUGHOUT THE PROCEDURE AND THE VALUES FOR THE PARTITION COEFICIENTS ARE SUBJECTIVELY ESTIMATED AND HAVE NOT BEEN DETERMINED EXPERIMENTALLY. EVEN WITH THESE DISADVANTAGES THE WASHOUT TECHNIQUES APPEAR TO AGREE WELL WITH OTHER TECHNIQUES FOR MEASURING REGIONAL BLOOD FLOW. AN IMPORTANT ADVANTAGE OF THE TECHNIQUE IS THAT REPEATED MEASUREMENTS CAN BE MADE IN THE SAME ANIMAL IF CATHETERS

TABLE D-3 (CONTINUED)

TESTS EMPLOYED	SPECIES OF ANIMALS USED	SUBSTANCES TESTED	REFERENCES	COMMENTS
KRYPTON-85 (^{85}Kr) AND XENON-133(^{133}Xe) WASHOUT				ARE PERMANENTLY IMPLANTED IN THE RENAL ARTERY. REPEATED MEASUREMENTS HAVE BEEN MADE IN CATHETERIZED DOGS FOR AS LONG AS ONE YEAR.
HYDROGEN WASHOUT	RATS	GLYCEROL, MERCURIC CHLORIDE	CHURCHILL ET AL., 1977	THIS IS A NON-RADIOACTIVE WASHOUT TECHNIQUE IN WHICH TISSUE HYDROGEN CONCENTRATIONS ARE MEASURED USING SURGICALLY PLACED PLATINUM ELECTRODES.
RADIOACTIVE MICROSPHERE-UPTAKE BY GLOMERULAR CAPILLARIES	RATS, DOGS	HYDROPENIA (1) SHALINE DIURESIS (2)	AUKLAND, 1980; BLANTZ ET AL., 1971; HSU ET AL., 1976; KATZ ET AL., 1971; MALIK ET AL., 1976; PREUSS, ET AL., 1975; SAINI AND SOMANI, 1979; SLOTKOFF ET AL., 1971; STEIN ET AL., 1973; YARGER ET AL., 1978	IN THIS TECHNIQUE, PLASTIC RADIOLABELED MICROSPHERES ARE INJECTED AS A BOLUS IN THE LEFT VENTRICLE OR THE ROOT OF THE AORTA AND ARE ENTRAPED IN THE RENAL TISSUE. THE QUANTITY OF MICROSPHERES ENTRAPED IN ANY REGION OF THE KIDNEY IS A FUNCTION OF BLOOD FLOW TO THAT REGION. THE ANIMAL IS TERMINATED: THE RENAL CORTEX IS REMOVED, SLICED AND COUNTED USING A LIQUID SCINTILLATION COUNTER. THIS PROCEDURE IS AN ALTERNATIVE TO THE INERT-GAS WASHOUT PROCEDURES FOR DETERMINING REGIONAL PLASMA FLOW RATES. TWO DIFFERENT RADIOLABELED MICROSPHERE INJECTIONS CONTAINING DIFFERENT RADIOLABELS CAN BE USED: ONE BEFORE TREATMENT

TABLE D-3 (CONCLUDED)

TESTS EMPLOYED	SPECIES OF ANIMALS USED	SUBSTANCES TESTED	REFERENCES	COMMENTS
RADIOACTIVE MICROSPHERE-UPTAKE BY GLOMERULAR CAPILLARIES				WITH A NEPHROTOXIN FOR A CONTROL AND ONE AFTER TREATMENT AND THE ALTERATIONS IN REGIONAL BLOOD FLOW DUE TO DAMAGE CAN THEN BE ASSESSED.
(1) CONDITION CHARACTERIZED BY WATER DEFICIENCY (2) CONDITION CHARACTERIZED BY INCREASED URINE SECRETION				

APPENDIX E

BIOCHEMICAL DAMAGE INDICATORS

TABLE E-1

BIOCHEMICAL DAMAGE INDICATORS: URINARY ENZYME ACTIVITY

TESTS EMPLOYED	SPECIES OF ANIMALS USED	SUBSTANCES TESTED	REFERENCES	COMMENTS
γ-GLUTAMYL TRANSPEPTIDASE, ACID PHOSPHATASE, ALKALINE PHOSPHATASE, GLUTAMIC OXALOACETATE TRANSAMINASE, GLUTAMIC PYRUVIC TRANSAMINASE, LACTIC ACID DEHYDROGENASE, β-GALACTOSIDASE, β-GLUCOSIDASE, β-GLUCURONIDASE, α-FRUCTOSIDASE, α-MANNOSIDASE, GLYCOSIDASES, N-ACETYL-β-GLUCOSAMINADASE, ISOCITRATE DEHYDROGENASE, SUCCINIC DEHYDROGENASE, β-N-ACETYLHEXOSAMINIDASE, MALTASE, URINARY LYSOZYME, MONOAMINE OXIDASE, CYTOCHROME C OXIDASE, δ-AMINOLEVULINIC ACID SYNTHETASE, MALATE DEHYDROGENASE, MURAMIDASE, KININOGENASE, KININASE, AMYLASE, LEUCINE AMINOPEPTIDASE, ALDOLASE, LEUCINE AMINOPEPTIDASE	RATS, RABBITS, CATS, DOGS, SWINE, HUMANS (CHILDREN AND ADULTS)	MERCURIC CHLORIDE, CADMIUM, URANIUM NITRATE, METHYLMERCURY HYDROXIDE, CADMIUM CHLORIDE, URANYL ACETATE, URANYL NITRATE, GENTAMICIN, SODIUM CYANATE, POTASSIUM DICHROMATE, SODIUM CHROMATE, ETHYLENEIMINE, STAPHYLOCOCCUS AUREUS, SODIUM TETRATHIONATE, d-SERINE, X-IRRADIATION, 4-NITROPHENYL-ARSENIC ACID, ENPHROTOXIC SERUM, TRITOQUALINE, ANAPHYLACTOID COMPOUND, PARAQUAT, DIQUAT, METHYL MERCURIC CHLORIDE, CARBON TETRACHLORIDE TRITOQUALINE	AMADOR ET AL., 1963 a & b, 1965; ASSCHER AND NASON, 1960; BALAZS ET AL., 1963; BETT ET AL., 1959; BREEDIS ET AL., 1943; CAREY AND BUTTERWORTH, 1969; COONROD AND PATTERSON, 1969a; ELLIS ET AL., 1973a, b & c; EVAN AND DAIL, 1974; FOWLER AND WOODS, 1977; GAULT AND GEGGIE, 1969; GAULT AND STEINER, 1965; GOLDBARG ET AL., 1959; HOBBS AND AW, 1968; KEMP AND LAURSEN, 1960; KING ET AL. 1976; LOCK AND ISHMAEL, 1979; LUNSETH, 1960; MCGEACHIN AND HARGAN, 1956; NEW ET AL., 1962; NOMIYAMA ET AL., 1973, 1974; NUSTAD, 1970a & b; PATEL ET AL., 1975; PRESCOTT AND ANSARI, 1969; PRICE ET AL., 1970, 1971; RAAB, 1968, 1969a, b, c & d; RAAB ET AL 1969; ROBINSON ET AL., 1967a; SCHOENFELD, 1965; STROO AND HOOK, 1977a & b; WACKER AND DORFMAN, 1962; WACKER ET AL., 1964	URINARY ENZYME ACTIVITY DETERMINATION IS A RELIABLE METHOD FOR THE DETECTION OF TUBULAR INJURY AND FLUORIMETRIC ASSAYS OF URINARY ENZYMES ARE EASY TO PERFORM, ARE AUTOMATED AND CAN BE READILY INCORPORATED INTO A NEPHROTOXICITY SCREENING PROGRAM. CHANGES IN URINARY ACTIVITY PROVIDE A SENSITIVE INDICATOR OF EARLY RENAL TUBULAR DAMAGE AND SHOULD HAVE IMPORTANT APPLICATION IN A SCREENING PROGRAM. THE MAJOR DISADVANTAGE IS COLLECTING STERILE, UNCONTAMINATED URINE SAMPLES, DIALIZING THE SAMPLES AND PROPERLY PRESERVING THE SAMPLES FOR ANALYSIS.

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